

CORYNEBACTERIUM DIPHTHERIAE

- I. A CORRELATION OF RECORDED VARIATIONS WITHIN THE SPECIES.
- II. OBSERVATIONS AND DISSOCIATIVE STUDIES—THE POTENTIALITIES OF THE SPECIES.

By HARRY E. MORTON

1940

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF SCIENCE IN THE UNIVERSITY OF MICHIGAN.



CORYNEBACTERIUM DIPHTHERIAE

A CORRELATION OF RECORDED VARIATIONS WITHIN THE SPECIES¹

HARRY E. MORTON

*Department of Bacteriology, School of Medicine, University of Pennsylvania,
Philadelphia, Pennsylvania*

Diphtheria is one of the diseases either diagnosed or confirmed in the laboratory by the isolation and identification of the causative microorganism, therefore it is of great practical as well as theoretical importance that we know as much as possible concerning the range of variation and the dissociative behavior of the causative organism commonly referred to as "the diphtheria bacillus." The organism has been studied extensively, but often the investigations were confined to certain aspects while totally ignoring other aspects or even previous studies along the same lines. It is not surprising then to find in the literature numerous conflicting reports on the behavior of the diphtheria bacillus.

Our present pleomorphic concept of bacterial species is one which depicts order and not chaos within each species. For several bacterial species it has become possible to correlate many of the chemical and physical properties of the microorganisms with colony form. A preliminary review of the literature (Morton, 1935b) suggested that this is also possible in the case of the diphtherial species. It is from this point of view that the experimental work reported elsewhere (Morton, 1940) was carried forward and the following review prepared.

So-called dissociative studies of recent times have contributed to a better understanding of a bacterial species; and the variations already reported fall into a generally consistent pattern. In the case of the diphtheria bacillus, a consideration of the earlier

¹ From the Hygienic Laboratory, University of Michigan, Ann Arbor, Michigan and the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

reports of its variation shows that, on the whole, the isolated observations do not represent incongruities within the species but rather an orderly trend in the variation process. Many of the seemingly independent variations can now be correlated with variations in colony form. So evident is this that it is often possible to predict many of the properties of a culture by merely establishing the colony form to which it belongs. Despite the relatively short period in which the various culture phases have been recognized as such, there is present in the older literature much valuable information which, if rightly interpreted, contributes to our better understanding of the variation problem.

The review which follows endeavors to treat only of the nature and degree of the recorded variations of the diphtheria bacillus and the extent to which they may be correlated with the recognized colony forms. The characteristics or properties are considered in the following order: (1) Colony forms; (2) Appearance of growth in liquid media; (3) Stability of the cell suspensions in salt solution; (4) Virulence; (5) Toxigenicity; (6) Serological reactions; (7) Hemolysis; (8) Fermentation reactions; (9) Chromogenesis; (10) Reduction of potassium tellurite; (11) Cell morphology.

1. COLONY FORMS

The smooth (S), intermediate (SR), and rough (R) colony forms

Six years after Loeffler's original description of the colonies produced by the diphtheria bacillus, variations were reported by Klein (1890). Peters (1897) also noticed a variation in the colonies, but the first to give serious attention to colony variation of the diphtheria bacillus were Cobbett and Phillips (1897). In addition to describing "shiny" and "matt" colonies, Zupnik (1897) confirmed the observations of Cobbett and Phillips that the composition of the medium is very important in determining the colony form. Serum or glycerol agar do not differentiate the shiny and matt colonies, but the difference can be observed on ordinary agar. These findings were confirmed by Schick and Ersettig (1903); and numerous others have reported the occurrence of two distinct colony forms for the diphtheria bacillus.

Following Arkwright's (1921) coinage of the terms *smooth* and *rough* to describe the forms of the colonies produced by the intestinal group of microorganisms, and the application of these terms to the colony forms of other species, Scott (1923) named the two kinds of colonies of the diphtheria bacillus as smooth and rough. Cowan (1927) has claimed a separation of the Park No. 8 and of another strain into smooth and rough colonies by selective cultivation; however, one wonders if she had the pure S form, because her smooth colonies are described as "less raised and less dense" than the R colonies. The purity of her R form is also open to question, for it is stated to produce a smaller pellicle than the S. A very important observation by Miss Cowan is that in the development of the R form the organisms are seen to pass through a stage in which the colonies are, for the most part, very large, irregular and coarsely granular, and the individual bacilli are also larger and of a great variety of shapes, often showing branched forms. Parker (1928) mentions smooth and rough strains, but here again the findings are not in accord with what one usually thinks of as the actual smooth and rough colony forms. Okell (1930) was not able to confirm Cowan's work even with the same cultures used by the latter; but this is not at all unusual since cultures very often cannot be dissociated at will. Yü (1930) was able to dissociate the S colony form to what he regarded as the R but was unable to change the R back to the S. Viewing the pictures of his colonies, however, one wonders if he had the actual R form.

Study of the colony form of the diphtheria bacillus assumed a different aspect following the description by Anderson, Happold, McLeod, and Thomson (1931) of "gravis" and "mitis" forms. After routine laboratory examinations over a period of eight years, they concluded that there are two distinct, stable types of the diphtheria bacillus with which are associated the following characteristics.

Type gravis. The growth on potassium tellurite chocolate medium after 48 hours is gray or gray-black. The colony is like that of *C. xerosis*, but heavier. The cell morphology on media, other than Loeffler's, is that of a very short diphtheroid, usually without granules. On

Loeffler's medium the granules are sometimes well marked and at other times scanty. There is a pellicle and granular deposit in broth; and the organisms give rise to unstable suspensions in saline, are non-hemolytic, possess typical fermentation reactions (no acid with sucrose, acid with glucose, maltose, galactose and invariably with dextrin, starch and glycogen), and grow vigorously on the special chocolate tellurite medium. The organisms are associated with severe cases of diphtheria.

Type mitis. The growth on the potassium tellurite chocolate medium after 48 hours is black. The colony is like that of *C. hofmanni*, but finer and more translucent. The cell morphology is that of the textbook *C. diphtheriae*, the bacilli usually being long and the granules well marked. There is uniform turbidity in broth; and the organisms produce stable suspensions in saline, are hemolytic, give recognized fermentation reactions, (no acid with starch or glycogen and inconsistent results with dextrin), and are partially inhibited upon subculturing to the chocolate tellurite medium. The organisms are associated with mild cases of diphtheria. The type *mitis* is commonly non-virulent for guinea pigs, but the type *gravis* almost always shows some degree of virulence.

Later (1933), Anderson and his colleagues proposed a third, type *intermedius*, that was said to be associated with diphtherias of intermediate severity. Its description follows.

Type intermedius. The type of growth on potassium tellurite chocolate agar, serum agar, heated blood agar, or plain agar is fine. The colonies are flat with central knob and slightly crenated periphery. On potassium tellurite chocolate agar the colonies are very fine with slightly raised black center and thinner translucent periphery. There is granular growth in broth, which settles rapidly. Neither starch nor glycogen is fermented. The morphology is that of a barred diphtheroid with metachromatic granules variable but often poorly developed.

There are at least two serious faults in the above descriptions and assumptions. First, not much information is conveyed in a description which states that a colony form is like that of *C. xerosis* or of *C. hofmanni*, as bacteriologists have realized since the works of Arkwright (1921) and Hadley (1927, 1937) demonstrated that no microorganism has a single and distinctive colony

form. The color plates, however, convey a fairly good picture of the two colony forms, which Anderson, *et al.*, were attempting to describe. Second, it is highly questionable whether or not it is possible to associate slight variations in clinical manifestations with colony form. Menton (1932) and others believe that the terms "gravis" and "mitis" have no biological basis. Although Anderson, Cooper, Happold, and McLeod (1933), and Carter (1933) claim that the colony forms and fermentation reactions are stable over a long period of cultivation, such claims are not in strict agreement with our present conceptions of bacterial variation. It is not surprising then that other workers are in disagreement, notably Wright and Rankin (1932), and Menton, Cooper, Duke, and Fussell (1933). Evidence of variation of the types *mitis* and *gravis* is given by Christison (1933) who observed a type *mitis* strain change its manner of growth in broth from turbidity and compact sediment to that of a pellicle and granular sediment with the supernatant fluid remaining clear, by subculturing in broth of pH 7.8 at three-day intervals. In addition, Menton was able to transform the type *gravis* colony to the type *mitis* form by the addition of commercial antitoxin to the medium. The work of Robinson (1934) shows that the various type strains of *C. diphtheriae* are relatively stable in ordinary stock cultures, but that by the usual *in vitro* and *in vivo* methods employed to promote variation, these strains show marked variation in most of the type characteristics. These latter findings are in keeping with our concepts of the dissociative behavior of a bacterial species. Menton (1932), Murray (1935a), and others have observed that typical colonies of one form may have the biochemical characteristics of another colony form; and numerous workers, in addition to Robinson and Peeney (1936), have contributed evidence for the variations in type characteristics of the strains.

Not only is type *gravis* not always associated with severe cases of diphtheria, nor type *mitis* with mild cases, but occasionally both types are isolated from patients or found in stock cultures. Parish, Whatley and O'Brien (1932) found that the mortality rate in human beings is about the same for organisms of both

colony forms and that, over a large region, the type *gravis* strains have been reported to be as prevalent in mild cases as in severe cases. Although the range in virulence of type *mitis* strains for guinea pigs was found by Anderson, *et al.*, to be greater than that of type *gravis* strains, the latter showing more uniform virulence, only three of the 90 strains tested for virulence by these workers, (employing the intracutaneous method on guinea pigs), gave a very marked reaction and these were type *mitis* strains. Whatever the type, 50 per cent or more, of the strains produced a moderate reaction in the guinea pigs. Bearing in mind the work of Powell (1923), who found that 40,000 to 400,000 diphtheria bacilli are required for a minimal reacting dose in the intradermal method of estimating virulence, one may well hesitate to interpret a reaction to the test dose as doubtful, slight, moderate, marked or very marked, unless the dose is more sharply standardized. If administered either subcutaneously or intradermally into guinea pigs, the type *mitis* strains are as virulent as the type *gravis* strains (Parish, Whatley, and O'Brien). When administered either intravenously or subcutaneously into rabbits, the type *mitis* strains appear to be more virulent.

It is quite obvious from the data that no one particular colony form exemplifies the diphtherial species. Diphtheria bacilli are either virulent or nonvirulent, and, if virulent, this quality is not necessarily associated with any particular colony form as, for example, is the case with the pneumococcus. The quantitative differences between virulent strains of diphtheria bacilli can be explained on the basis of S-R variation.

Later and more detailed reports of rough colonies of the diphtheria bacillus are those by Whitley (1934), who found a "rough" form present in nose and throat cultures; by Morton (1935a, 1940) and Hobby (1935), who produced R forms by forced dissociation; and by Bisset (1938) who encountered R colonies in strains which were being repeatedly subcultured. Thus far, of all the descriptions of the rough colony forms, those by Morton and by Hobby appear to represent the ultimate rough form, in that they more nearly fulfill the criteria for a rough culture phase as described by Hadley. Following are the criteria for a rough,

or R, colony form, in general. The margin is very irregular; the surface is very flat, uneven and granular; the organisms grow as a granular sediment in broth and clump spontaneously in physiological salt solution. Evidence for this type of colony does not appear very often in the literature, in spite of the fact that workers have designated some of their colony forms as rough.

The usual criteria for the smooth, or S, colony form, in general, are: the margin is round and even; the surface is convex and smooth; the organisms grow in broth with a uniform turbidity, and produce a stable suspension in physiological salt solution. Workers prior to Baerthlein and Arkwright did not always search for these cardinal points, but from their meager descriptions one is able to recognize some of the colonies, undoubtedly, as of the smooth form. The first impression one receives from the description by Anderson, Happold, McLeod, and Thomson (1931) is that their type *mitis* colonies are the smooth form. This impression is verified when one examines the colonies by some special means of illumination, as did Murray (1935b).

During the transformation of the smooth to the rough colony form, the surface of the colonies becomes flattened, irregular and granular; and, as Cowan mentioned, the colonies also become larger. It is this intermediate form, SR (somewhat removed from the pure S), which produces the pellicle type of growth with sediment and clear supernatant, so often obtained with diphtherial cultures. One immediately recognizes the similarity of the type *gravis* colonies described by the English workers and the SR colony form.

From the description by Anderson, *et al.*, it appears that some of their intermediate forms, which could not be classified as either type *mitis* or type *gravis* colonies, represent, perhaps, a stage of the diphtherial organism somewhat removed from the SR stage and approaching roughness, whereas other forms in this group are suggestive of a dwarfed smooth colony. The term "intermediate", when used in this connection does not necessarily mean that such a colony is intermediate between those of types *mitis* and *gravis*. In the terminology employed in respect to smooth and rough colonies, the term "intermediate" signifies

that the colony has an appearance that is in between those of the smooth and the rough forms; the "intermediate" colony can be observed to arise from one form and proceed into the other.

When one reads the reports of the incidence of types *gravis* and *mitis*, and intermediate forms in various localities and epidemics, it is readily apparent that the predominating type is likely to vary from one locality to another and from one epidemic to another. If virulence is not necessarily associated with one particular colony form of the diphtherial species, as numerous workers have shown, then it is possible for one colony form to predominate in a given epidemic or in a certain locality and to be replaced by another colony form during another epidemic or in another locality, which, in fact, is substantiated by the findings of Frobisher (1939).

In no other species of microorganisms have colony forms been named according to the quantitative variations in clinical manifestations which they produce, and, from the number of discordant reports, this does not appear to be the most satisfactory basis for nomenclature for the diphtherial species. The inadequacy of the classification is exemplified by the necessity for the creation of sub-groups, as was done by Wright, Christison, Rankin, Pearson, and Cuthbert (1935), and Stuart (1938), who found no correlation between virulence, colony form, and the ability of a strain to ferment starch. It is surprising that earlier students of types *gravis* and *mitis* did not attempt to associate the various colony forms with S-R variation, a system of variation and nomenclature which has been found applicable and useful for hundreds of other bacterial species.

Dwarf colony form (D)

It is interesting that in the same year in which colonial variation of the diphtheria bacillus first received serious study, dwarf colony forms were encountered. It was only the larger forms, however, the smooth and rough colonies, which were studied in the years that followed. Cobbett and Phillips in their studies in 1897 described extremely minute colonies of the diphtheria bacillus. When 10 cultures of virulent diphtheria bacilli were

grown on gelatin, 6 of them yielded two varieties of colonies, designated as large and small colony types. Two cultures yielded only the small colony type and two the large type only. When separated into pure cultures, the large and small colony forms maintained for many generations their characteristic difference in size. From pure cultures of the large colonies, all the colonies in subsequent generations were of the same large type. Pure cultures of the small colonies, however, although continuing to produce small colonies for many generations, occasionally gave rise to colonies of the large type. One strain maintained constantly the property of producing only the small colony form. Cobbett and Phillips at first were inclined to ascribe this phenomenon to their failure to separate completely the two varieties and not to a tendency to variation; but later they ruled out the possibility of impure cultures. The appearance of the two colony forms on gelatin was the only instance in which the strains varied, the strains were alike in virulence, microscopic appearance, and in their manner of growth in broth and upon media other than gelatin. These small colonies were extremely minute, often scarcely visible, and seldom as large as a half-millimeter in diameter.

According to the type of growth on trypsin-serum agar, Parker (1928) temporarily classified diphtherial cultures into heavy and light growers. The heavy growers were further classified into R-heavy and S-heavy growers. The light growers were not as easily differentiated from the heavy growers on serum agar in which the serum had not been trypsinized nor on plain agar. The light growers were in every case (16 strains) toxigenic. These light growers described by Parker, appear similar to the small colony forms described by Cobbett and Phillips, and may well be the forms which at the present time are referred to as dwarf (D) forms.

The "intermediate" type (of Anderson *et al.*) when plated from broth cultures was observed by Christison (1933) to yield frequently two kinds of colonies. One kind was irregular, granular, and flat with a central knob, which is suggestive of an sR colony form, and the other was much darker, glistening and

entire, but smaller and more convex than type *mitis* colonies of the same age. Since Christison made no statement as to the exact size of the colonies, it is difficult to interpret this colony form, but there is a possibility that it might be the dwarf colony form of the diphtheria bacillus.

Bacilli from type *gravis* colonies have also been observed to give off small-colony variants. Erzin (1936) describes two forms of growth for this type: typical type *gravis* colonies and "zarte" colonies. On blood agar the "zarte" colonies are round, convex, small, and hemolytic. On Clauberg's medium the colonies are soft, delicate, and tiny. On the medium of Gundel and Teitz the colonies are very small, delicate, appearing scarcely visible through a magnifying glass, and sometimes convex and shiny. On Loeffler's medium and in broth the organisms produce "typical growth for diphtheria." Upon the same five media, the type *gravis* or "typical" colonies produce "typical growth." Incubation of a broth culture of the "zarte" form at 37°C. gives rise after 10 days to a mixture of "zarte" and type *gravis* colonies. Incubation of a broth culture of type *gravis* at 37°C. gives rise after 34 days to a mixture of the two forms. The "zarte" form produces toxin but is less virulent for guinea pigs than type *gravis*.

Morton (1935a) reports small colony variants of the diphtheria bacillus, which are designated dwarf or "D" colonies. The small colony variants were produced by the aging of broth cultures of a Park No. 8 strain. The organisms are pathogenic for guinea pigs, are toxigenic, and ferment glucose and dextrin but not sucrose. They are agglutinated by immune serum prepared by immunizing rabbits against the parent culture of the Park No. 8 strain. In two filtration experiments, the organisms were not found to pass through a Berkefeld N filter. Hobby, in 1935, reported that her R type colony from a recently isolated diphtherial strain (RB-2T) either remained as such or was transformed to a minute or "G" colony.

Two forms of small colonies were observed by Mittag (1937) in addition to the commoner large forms. The small, irregular colonies, ("kleine, zarte, gekerbte Kolonien") appear to be the sR form, and the small, delicate, round colonies ("kleine, zarte,

runde Kolonien") appear to be the dwarf colonies. The organisms were pathogenic for guinea pigs.

Our review shows that small colony forms have been described for the diphtheria bacillus on at least six different occasions. The ability to produce an extremely small colony form, in addition to the more common large colony forms, is not a unique characteristic of the diphtherial species but seems to be a property inherent in many bacterial species. Similar forms have been described for the following: *Bacillus megatherium* (Rettger and Gillespie, 1933), *B. mesentericus* and *B. vulgatus* (Flynn and Rettger, 1934), *Clostridium welchii* (Roe, 1932, 1934), *Diplococcus pneumoniae* (Dawson, 1928; Eaton, 1934), *Eberthella typhosa* (Fromme, 1911; Eisenberg, 1914), *Hemophilus pertussis* (Kimball, unpublished data), *Lactobacillus acidophilus* (Kopeloff, 1934), *Neisseria gonorrhoeae* (Raven, 1934), *Pseudomonas fluorescens* and *Ps. pyocyanea* (Eisenberg, 1914), *Salmonella aertrycke* and *S. schottmuelleri* (Fürth, 1922; Koser, 1930), *S. suispestifer* (Orcutt, 1923), *Shigella equirulis* (Edwards, 1931), *S. dysenteriae* Shiga (Arkwright, 1921), *S. sonne* (Koser and Styron, 1930; Koser and Dienst, 1934; Chinn, 1936), *Staphylococcus aureus* (Swingle, 1934) and a group C hemolytic streptococcus (Morton and Sommer, unpublished data). In many of these cases the small colony forms were called dwarfs, and in other cases they were called "G" colonies in spite of the fact that they did not satisfy the criteria for the G type colony. If more care had been exercised in the classification of small colony forms, less confusion would now exist concerning the G forms. These dwarf forms are not at all unusual. One notices that they may occur under normal conditions of cultivation, but that their ratio to the larger colony forms is quite small. Frequently the dwarf colony forms are produced under the influence of aging or by growth in the presence of lithium chloride. One also recognizes that they are produced from cultures that are showing some evidence of, or a tendency toward, the rough phase. When practically the same phenomenon has been observed and described for at least 20 bacterial species, representing more than 12 different genera, it would seem to warrant recognition as a definite phase in bacterial variation.

Gonidial colony form (G)

In addition to the small colony forms mentioned above, which have been described by various workers since 1897, a few of the observations warrant special attention, because a small colony (G) form was obtained under special conditions of cultivation, and because it represents growth from *filterable* elements of the bacteria. In this respect the G colony form is unique. Hauduroy (1927) obtains such colonies from filtrates of diphtherial cultures by serially culturing the filtrates on solid media. This is accomplished by seeding a few drops of the filtrate on an agar plate. After suitable incubation the surface of the plate is washed with a few drops of sterile broth, and the washings are transferred to another fresh, sterile plate, this process being carried forward in series. Smith and Jordan (1930) describe growths from 11 of 19 filtrates of aged diphtherial cultures after serial transfers of the filtrates as recommended by Hauduroy. Hadley and Richardson (Hadley, Delves and Klimek, 1931) and Morton (1932) also report small colonies (designated as G colonies) from filtrates of diphtherial cultures.

Since many workers have been erroneously calling all small colonies G colonies through failure to establish that the organisms therefrom fulfill the necessary requirements, these criteria as proposed by Hadley (1931) will be reiterated. They are as follows:

"1. Visible signs of culture development appear in the filtrate, or in subcultures from it, only after a considerable delay, perhaps after from 6 to 12 days, or even after a longer period in the case of some species.

"2. Colonies on agar plates are very slow in appearing, perhaps after from 24 hours to 8 days, and the first colonies to arise are of minute size, having an average diameter of 0.2 mm. or less at the end of 96 hours on a favorable medium present in sufficient depth in the plate.

"3. The morphologic type of the organisms present in the filtrate, or in subcultures or colonies, is composed mainly of minute coccus forms, granular bodies and delicate skeins or filaments, with a suggestion of diplococcus or streptococcus arrangement (Giemsa staining); also occasional coccobacilli.

"4. In organisms the 'normal' form of which is acid-fast or gram-positive, the staining reaction of the recovered culture elements is temporarily reversed or variable.

"5. The biochemical and serologic reactions of the recovered cultures are different from those of the parent culture, and virulence, toxicity, and susceptibility to the homologous bacteriophage are altered."

Of course, contamination is ruled out by eventually regaining the original culture form after it has gone through its filterable stage.

Mucoid colony form (M)

Moist colonies have been described for the diphtheria bacillus, but Hobby (1935) was the first to name a mucoid phase of the diphtheria bacillus. It has not yet been demonstrated, however, that diphtheria bacilli in the mucoid phase possess a capsule nor that they possess more of the type-specific substance than when in any other colony phase.

Summary. Thus, during the period of more than half a century that has elapsed since the original observations of the colonies produced by the diphtheria bacillus, many colony forms have been noted. These various colony forms were either described, or may now be interpreted, as the mucoid (M), smooth (S), intermediate (SR), rough (R), dwarf (D) and gonidial (G). Photographs and additional detailed descriptions of these different culture phases will be found in the paper by Morton (1940). The terms "mitis," "gravis," and "intermedius" as applied to the colony forms of the diphtheria bacillus do not truly designate the clinical manifestations which were originally thought to be associated with these cultural phases. Since the form of colony to which these terms originally referred can be described adequately by the older and accepted terminology (smooth and rough colony forms), there appears to be no need for the continuance of the terms "mitis," "gravis" and "intermedius" in that respect.

2. APPEARANCE OF THE GROWTH OF SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY FORMS IN LIQUID MEDIA

The manner of growth of a strain in broth often conveys as much information relative to the culture phase as does the appear-

ance of the colonies on solid media. In addition to confirming the observations of Roux and Yersin (1888) that diphtheria bacilli grow in broth as fine granules which adhere to the walls of the vessels, Brieger and Fraenkel (1890) observed that in some cases the bacilli grow to produce a homogeneous and uniform turbidity. The first indication of a correlation of such types of growth with differences in colony form in the diphtherial species came from the work of Baerthlein (1913) and of Bernhardt (1915) who reported that cells from a large, moist colony form grew with an even turbidity, whereas those from a fine, translucent, bluish, and brittle colony form grew only as a heavy, granular precipitate. The large, moist colony form described by Baerthlein produced a black color when grown on tellurite medium; and the fine, translucent, bluish form of colony produced only a light brown color. This represents the first indication that appearances on tellurite medium, in addition to the manner of growth in broth, may also be associated with colony form. Riemsdijk (1914) described three types of growth in broth, all of which can now be correlated with colony form: (1) granular growth on the bottom of the tube, associated with the rough colony form; (2) homogeneous clouding, associated with the smooth colony form, and type *mitis* colony described by the English workers; and (3) occasional pellicle formation, associated with the intermediate (SR) colony form and type *gravis* colony. The mucoid form is described by Hobby as growing with a uniform turbidity, similar to that produced by the S form. Neither the M, S, nor R forms grow in a granular pellicle (Hobby). These isolated observations have been confirmed by Morton (1940), who found in addition that the D form grows with a very faint turbidity, which is often finely granular. The G form often fails to impart visible signs of growth in filtrates for a considerable time. When visible signs of growth do occur, it is as a very fine turbidity with a ropy sediment. The statement in Bergey's manual that the diphtheria bacillus grows in broth as a "fine, granular deposit on sides and bottom of tube, forming a thin, fragile pellicle on neutral medium" is inadequate.

3. STABILITY OF THE CELL SUSPENSIONS IN SALT SOLUTION

The spontaneous clumping of diphtheria bacilli in physiological salt solution has often been very disconcerting. Indeed, Priestley (1911) reported that an agglutination test for the identification of *C. diphtheriae* was impractical since 12 out of 15 strains tested by him clumped spontaneously. The addition of glycerol, formalin, various salts, etc., failed to prevent the spontaneous clumping. Early, unsuccessful attempts were made to associate the property of remaining in uniform suspension in physiological salt solution with virulence or toxigenicity. Scott (1923) reported that such spontaneous clumping is not consistently associated with either the presence or absence of toxigenicity; therefore, it is not surprising that Okell (1930) and Jones (1930) were unable to find any significant relationship between agglutinability in salt solution and virulence. Although Scott found no complete association between roughness of growth and sensitiveness or insensitiveness to electrolytes, more recent studies have shown that sensitiveness to electrolytes is associated with colony form. Yü (1930) and Morton (1940) both found that the smooth colony form yields a uniform suspension in physiological salt solution. (The "mitis" form, which may be interpreted as a smooth colony form, gives uniform suspensions). The rough colony form was found to give granular and unstable suspensions. In a comparative study of this property among the S, SR, R, and D colony forms of diphtheria bacilli (Morton, 1940), it was found that the S and D forms give uniform suspensions in salt solutions in a concentration of 0.2 to 8 per cent, organisms in the R form clump spontaneously, and those intermediate between the S and R give indeterminate results.

4. VIRULENCE

It seems to be agreed that diphtheria bacilli, as identified by colony form, fermentation reactions, agglutination tests, etc., may be either virulent or non-virulent for guinea pigs, *i.e.*, the microorganisms, when injected into guinea pigs, may or may not bring about the death of the animals. The strains, whether

virulent or non-virulent, undergo the same variations in colony form. Earlier workers believed that diphtheria bacilli vary in virulence, ranging from non-virulent to highly virulent. Recent workers employing single-cell cultures and the intradermal method for estimating virulence find that diphtherial cultures are either virulent or non-virulent, none being intermediate (Powell, 1923; Dudley, 1925 and Okell and Parish, 1926). The work of Powell, in addition to showing that the virulence of diphtheria bacilli for guinea pigs varies only within narrow limits, showed that 40,000 to 400,000 organisms are required for a minimal reacting dose by the intradermal method, whereas 14 million to 140 million, or 35 times as many organisms are required when employing the subcutaneous method for estimating virulence. This, while establishing the superiority of the intradermal over the subcutaneous method, still leaves a rather wide latitude in the effective number of microorganisms.

Powell found that single-cell strains have the same degree of virulence as their respective parent cultures and different single-cell strains derived from the same single-cell strain are equally virulent for guinea pigs. However, since Roux and Yersin (1890) first reported that virulent diphtheria bacilli may give off non-virulent variants, other observers have noted the same phenomenon. In view of the fact that other species of virulent microorganisms give off non-virulent variants, the same process may be readily accepted for the diphtheria bacillus. Reports of the reverse process, that of non-virulent variants reverting to virulent forms, are far less common, and some believe that it cannot take place. Trumpp (1896) claimed to have transformed an avirulent strain into one possessing virulence by injecting the culture into guinea pigs along with sublethal doses of toxin. Morton (1940) reported that after two cultures of the virulent Park No. 8 strain had aged in ampoules 132 and 140 days, respectively, the organisms produced S colonies on solid medium whereas the strain originally was of the SR colony type. Upon five occasions, cultures from the S colonies were non-virulent for guinea pigs. After 14 generations on plain or blood infusion agar, one of the S strains killed guinea pigs in doses of 37,000,000 or-

ganisms administered intraperitoneally. An explanation for this reversion might be that during the physiological youth of the culture, it had not attained its maximum toxin-producing properties, just as was shown (Morton, 1940) that during the physiological youth of a diphtherial culture great variations in morphology may take place.

A study of colony forms has not contributed anything toward an explanation for virulent and non-virulent forms of the diphtheria bacillus. Cobbett and Phillips (1897) noticed no difference in virulence between the strains derived from their various colony forms. The dissociative studies by Hobby were made, unfortunately, on a strain which did not possess virulence. In the studies by Morton on virulent strains, the various colony forms (S, SR, R, and D) retained their pathogenicity for guinea pigs. Yü (1931) reported that his S, virulent form, isolated during the acute stages of diphtheria, was followed during convalescence by non-virulent S and R forms. Nevertheless, virulence is not associated with colony form as Yü thought it to be. This is evident from the studies by Cobbett and Phillips and by Morton, and is borne out by the reports cited in the next section on toxigenicity, where there are listed the results obtained from the use of culture filtrates instead of whole cultures.

It is rather instructive to examine the most recent description of the pathological effects of diphtheria bacilli in man as observed by McLeod, Orr, and Woodcock (1939) on the morbid anatomy in *gravis*, *intermedius*, and *mitis* diphtherias from a series of 51 necropsies. Their study indicates that an infection with type *mitis* (S form) diphtheria bacilli is of less serious menace to the individual than an infection with organisms of type *gravis* (SR form). In acute cases the primary lesions caused by types *gravis* and *intermedius* are differentiated from those of type *mitis* (S form) on the following points: much less superficial membrane in the fauces and less tendency to extend into the intrathoracic air passages; the membrane less firm in texture; deeper penetration of the tissues of the inflamed parts and greater involvement of the cervical lymph glands and surrounding tissues; and selective involvement of the tonsils and the deeper tissues at this site.

The most frequent causes of death were (a) respiratory obstruction as the result of membrane formation, which was more frequent with type *mitis*; and (b) toxic effects on the viscera, especially the heart and kidneys, which were more frequent with types *gravis* and *intermedius*. Thus, although virulence is not associated with colony form, the above evidence suggests that there may be a correlation between the colony form and certain pathologic characteristics.

Since the type *gravis* usually ferments glycogen in the test tube and type *mitis* does not, Knox and Passmore (1938) reasoned that the fermentation of glycogen might be the explanation for the greater virulence of type *gravis* within the human body. However, their finding by metabolic studies that both forms utilize glycogen eliminated this possible explanation. Povitzky, Eisner, and Jackson (1933) produced death in guinea pigs, weighing about 300 gm., in 24 to 48 hours with a Park No. 8, a type *mitis* and a type *gravis* cultures, employing the growth from $1\frac{1}{3}$, $\frac{9}{10}$, and $\frac{1}{3}$ veal-agar slants, respectively. Their explanation for the fact that the type *gravis* culture was more virulent than the others was that it probably produces toxin more readily in the animal body. Their *in vitro* results, however, do not bear out this reasoning, for in 48 to 72 hours, cultures from the above three strains contained 550, 350 and 200 M.L.D. of toxin per ml., respectively. Their *in vivo* results, in view of the findings of McLeod, Orr, and Woodcock, may be explained from another angle.

The vital question of why certain strains of diphtheria bacilli are virulent for man, guinea pig, or other animals, while other strains are not, is still unanswered. The property of virulence is not associated with colony form nor is toxigenicity the complete explanation. Perhaps careful chemical or metabolic studies will bring forth the long awaited answer.

5. TOXIGENICITY

In contrast to the previous section, wherein results of infection of the animal body with the various colony forms of the diphtheria bacillus were discussed, this section is concerned primarily with

the ability of these organisms to elaborate a toxin, as judged by testing the cell-free culture filtrate. Many of the technical details concerned with the production of diphtherial toxin have been described by Andrewes, *et al.* (1923). Investigators agree that diphtheria bacilli, as identified by colony forms, fermentation reactions, agglutination tests, etc., may or may not elaborate the specific exotoxin. Crowell (1926), working with a single-cell strain, observed that it is possible for a toxigenic strain to give off both toxigenic and non-toxigenic descendants. He, like others, never observed a non-toxigenic strain to regain its toxigenicity.

Toxigenicity is not necessarily associated quantitatively with virulence. It is generally known that the Park No. 8 strain, which produces one of the most potent diphtherial toxins, is not one of the most virulent strains. The work of Povitzky, Eisner, and Jackson (1933) showed quantitatively that the virulence and toxigenicity of different strains may vary independently of one another.

Toxigenicity is not associated with any serological type. Havens' (1920) report that the toxins elaborated by two serological types were not identical, created considerable consternation, for bacteriologists and clinicians had taken it for granted that there is but one diphtherial toxin. This report by Havens, however, was disproved by Paxson and Redowitz (1922), Hartley (1923), and Park, Williams, and Mann (1922). The latter workers found that the toxins elaborated by organisms of the various serological types are qualitatively, and from the practical standpoint, quantitatively alike.

It has been demonstrated that toxigenicity is not associated with colony form. The smooth and matt colony forms of Schick and Ersettig (1903) produced toxins which were neutralized by diphtherial antitoxin. Although Cowan (1927) and Yü (1931) claimed that their R strains failed to produce toxin, Povitzky, Eisner, and Jackson (1933) found that both types *mitis* and *gravis* produce toxin; and more recently Morton (1940) reported that the S, SR, R, and D colony forms produce toxin.

No matter in what serological group or colony form a diph-

theria bacillus may be, the toxin, if it is produced, is one and the same. There are, however, differences in the rate of production and the amount produced. The reason why certain strains of diphtheria bacilli are able to elaborate the exotoxin and other strains are not, is still unanswered. Dissociative studies and investigations into the antigenic structure of the bacterial cell have thus far failed to reveal the answer. Perhaps a study of the enzyme systems of the diphtherial cell will furnish an explanation.

6. SEROLOGICAL REACTIONS

Early investigators soon learned that there is no relationship between the agglutinability and the virulence or toxigenicity of diphtheria bacilli. Moreover, the agglutination reaction has failed thus far to reveal any changes in cellular antigens of virulent diphtheria bacilli when the bacilli become non-virulent. Also, it has not been possible to discover a diphtherial agglutinating serum capable of agglutinating a pseudo-diphtheria bacillus or a diphtheroid. Nor does a diphtherial agglutinating serum agglutinate all strains of diphtheria bacilli. The more this species is subjected to serological study, the greater is the number of serological types that are discovered. Lipstein in 1903 recognized four definite serological types of *C. diphtheriae*; and Durand (1918, 1920) reported five and a group of heterologous strains. Havens (1920) temporarily complicated matters by reporting only two serological groups but these findings were soon disproved by Bell (1922) and by Park, Williams, and Mann (1922). The latter found at least five serological types. Smith (1923) reported seven serological types; and Robinson and Peeney (1936), in a study of 739 strains, encountered a fifth type in addition to Ewing's (1933) four. Eagleton and Baxter (1923) found ten types, as did Sia and Huang (1939). Murray (1935c) reported eleven types and a group of unclassifiable strains. Undoubtedly more will be reported in the future.

Practically the same results have been obtained by agglutinin-absorption as by the agglutination test. The agglutinin-absorption procedure enables more strains to be classified because

spontaneous agglutination of the strains does not interfere with the text.

Only recently has the question of serological types been approached from a different angle of investigation, namely, by the attempt to isolate the chemical substance responsible for type-specificity. Wong and T'ung (1938) reported at first that the polysaccharides of *C. diphtheriae* are shared by types *mitis*, *gravis*, *intermedius*, and by an avirulent strain, and later (1939) that the polysaccharides are group-specific. It is quite possible that Wong and T'ung selected cultural variants of the same serological type for these studies. After Sia and Huang (1939) found different cultural forms within the same serological type, Wong and T'ung in their later work (1939, 1940) selected strains from different serological types, instead of from different cultural forms, for their chemical studies. As a result, they were able to isolate chemically a substance which reacts only with serum prepared against the homologous strain. To date the preliminary findings of Wong and T'ung are that the cellular constituent of the diphtherial cell which is responsible for serological type-specificity is an alkali-soluble protein that is heat-labile, and convertible to a group-specific protein by heating at 56°C. for 30 minutes.

In 1903 Schick and Ersettig reported that organisms from their smooth and matt colonies are agglutinated by the same anti-serum; and additional studies along this line by Morton (1940) showed that organisms in the S, SR, R, and D colony forms react similarly against type-specific serum. Single-cell strains from different colony cultures have the same agglutinative reaction as the parent strain (Powell, 1923).

The attempt on the part of the English workers to identify their types *mitis* and *gravis* by fermentation reactions and antigenic types appears to be impractical. It has been shown that microorganisms of one colony form may have the fermentation reactions of those of the other colony form. This, moreover, has been verified by studies in metabolism which reveal that microorganisms of both colony forms possess similar enzyme systems. Furthermore, it is not in keeping with our general

knowledge of bacterial species for different serological types within the species to be characterized by distinctive colony forms. In their investigations on the chemical substances within the diphtherial cell, Wong and T'ung found that a type-specific substance is present in strains of different cultural reactions, but it is present only in strains of the same serological type.

Inasmuch as the organisms in at least one of the major colony forms of the diphtherial species clump spontaneously in physiological salt solution and a fair percentage of diphtherial cultures encountered in nature give unstable suspensions, agglutinin-absorption or chemical fractionation of the organisms and precipitin tests, of necessity, have to be employed for serological typing. The chemical studies begun only recently by the Chinese workers should prove to be of great value. The early report that the bacilli from smooth and matt colonies are agglutinated by the same serum, and the preliminary finding that bacilli in the S, SR, R, or D colony forms behave similarly towards type-specific sera indicates that type specificity is not associated with a particular colony form, as is the case with *Diplococcus pneumoniae*.

7. HEMOLYSIS

It has been known since the report of Eijkman (1901) that diphtherial cultures are able to hemolyze red blood corpuscles, but that the hemolytic power is variable. The hemolytic substance is present in living cultures only, is destroyed by heat, and is non-filterable (Schwoner, 1904; Wesselow, 1914; Costa, Troisier, and Dauvergne, 1918). Practically all investigators have found that the hemolytic property in corynebacteria is associated only with the diphtherial species; strains of *C. xerosis* and pseudo-diphtheria bacilli lack this property. Red blood cells from different species of animals vary in susceptibility in the following decreasing order: rabbit, guinea pig, goat, dog, horse, and human (Schwoner, 1904; Goldie, 1933).

There is no correlation between the hemolytic power and the toxigenicity of a strain (Maunu, 1914; Heeren and Megrail, 1930), nor virulence nor the agglutination reaction (Hammer-

schmidt, 1924). Anderson, Happold, McLeod, and Thomson (1931) reported their type *mitis*, which we now know to be the smooth colony form, as hemolytic and their type *gravis*, which may be interpreted as an SR colony form, as non-hemolytic. The work of Christison (1933) brought out more strikingly the correlation of hemolytic power with colony form. She found that the rough strains derived from type *mitis* (smooth) colonies either fail to hemolyze red blood cells or give only faint traces of hemolysis. Smooth strains derived from type *gravis* colonies lyse red blood cells more frequently than do the rough strains.

In a comparative study of the hemolytic activity of the various colony forms, Morton (1940) reported that organisms in the S colony form are hemolytic, less so in the R, and the dwarf forms are non-hemolytic.

8. FERMENTATION REACTIONS

Andrewes, *et al.* (1923) in their monograph on diphtheria give a historical survey of the development of fermentation tests for diphtheria bacilli and the diphtheroids. All workers agree that *C. diphtheriae* ferments glucose with the production of acid only, and that dextrin is usually fermented. Sucrose is usually not attacked; but Martin (1898), Cary (1917), Durand (1921), Fitzgerald and Doyle (1923), and Wright and Rankin (1932) have reported instances in which diphtherial strains have fermented sucrose. However, in view of the rarity of sucrose-fermenting strains of diphtheria bacilli, the readiness with which sucrose solutions may become altered during sterilization by heat or simply through the aging of aqueous solutions, and the diversity of media and pH indicators employed in fermentation tests, it might be well for this property to be reinvestigated by careful metabolic studies.

Schick and Ersettig (1903) reported that organisms from both their smooth and matt colonies fermented glucose; and more recently Cowan (1927) and Yü (1930) reported that their smooth and rough colony forms had the same fermentation reactions, the only difference being that the R form required a longer time to bring about the reaction. Judging from the descriptions or

photographs submitted by Cowan and Yü, it is not certain that they were working with the true S and R forms. At any rate, organisms in the S, SR, R, and D colony forms have the same fermentation reactions (Morton, 1940). Organisms from SR colonies are the most rapid fermenters, whereas the D forms require the longest period of time to bring about the production of acid. The differences between the various colony forms are quantitative rather than qualitative, a condition which is usually the case with the various colony forms within a species.

Anderson, *et al.*, (1931) observed qualitative differences in the fermentation reactions for different colony forms of the diphtherial organism. They reported inconsistent results with type *mitis*, while type *gravis* invariably fermented dextrin. Sharper differences, however, were encountered with starch and glycogen, which were fermented only by type *gravis*. The more vigorous fermentative activity of organisms in the SR colony forms (Morton) may explain the greater frequency with which type *gravis* attacked dextrin. Not always does type *mitis* (S form) fail to attack starch and glycogen, for Carter (1933) reported 1.17 per cent of the cultures from 510 positive swabs as being aberrant in that starch and glycogen were fermented but that the organisms grew in broth with a turbidity and no pellicle, a condition which is very suggestive of the S form. Robinson and Peeney (1936) also observed strains which were smooth and fermented starch, and strains which in other cultural respects resembled type *gravis* but failed to ferment starch. Ewing (1933) found two of 106 strains that fermented starch and produced type *mitis* colonies. Moreover, Wright and Rankin (1932) reported that none of their strains isolated from 50 cases of diphtheria fermented starch. They also found that one type *mitis* and two intermediate strains in addition to the type *gravis* strains fermented glycogen.

From the foregoing, one must conclude that the different colony forms of the diphtherial organism can not be differentiated definitely by qualitative differences in the usual fermentation tests. The clinching evidence for this decision would be metabolic studies with suspensions of the bacterial cells, and so far as the author is aware the only metabolic studies planned to clarify

this point have been those of Passmore (1938) and Knox and Passmore (1938). The latter workers found that there is no significant difference between the oxygen consumptions per hour per mg. dry weight of bacterial suspensions of types *mitis* and *gravis* when employing glucose or glycogen as the substrate. On the other hand, when tested in the usual way with glycogen in fermentation tubes, type *gravis* produces a strongly acid reaction, while type *mitis* remains neutral and, in addition, shows no utilization of the glycogen. According to Knox and Passmore, the discrepancies in results obtained when using fermentation tubes are accounted for by the differences in aeration within the tubes.

The property of utilization of carbohydrates by the diphtheria bacillus needs to be reinvestigated by more accurate metabolic studies. The great variations in manner of growth in liquid medium unquestionably account for great differences in aeration within fermentation tubes. The studies by Passmore show that enzymes for breaking down carbohydrates, when present in only small amounts, may be completely suppressed in organisms during their growth on nutritive media. At present there is no evidence substantiating the claim of Anderson, *et al.*, that the colony forms can be differentiated by qualitatively different fermentation reactions. The diphtherial organisms behave like other bacteria in that the various colony forms have the same fermentation reactions, though there are, perhaps, quantitative differences.

9. CHROMOGENESIS

Numerous workers have observed the production of a pigment in diphtherial cultures. Andrewes *et al.* (1923) state that this property is probably more dependent upon the composition of the medium than upon the organism, but at the present time it is not known what is responsible for the pigmentation which is observed occasionally. A yellowish color was observed by Zupnik (1897), by Cobbett and Phillips (1897), and by Morton (1940). Baerthlein (1913) and Hadley (1927) observed the yellowish color among R variants while Baerthlein (1918) and

Ewing (1933) observed it among S strains. It is thus evident that pigmentation is not associated with any particular colony type, the pigmentation being observed in the S and R forms with about equal frequency. A citron-yellow color was reported by Przewoski (1912) and by Bernhardt and Paneth (1913) to be quite common. Heinemann (1917) found diphtherial cultures taking on a yellowish-brown color, as did Bernhardt (1915) and Hill (1903); the latter also reported a pink color.

No investigations were made upon the pigments until Smith (1930) observed in filtrates of diphtherial cultures a pigment which he identified as a porphyrin. He was not able to establish any relationship between the rate of formation of the pigment and the rate of toxin formation. Although Coulter and Stone (1931) reported a direct relationship between the biological titer of culture filtrates and porphyrin content, the work of Wadsworth, Crowe, and Smith (1935) suggests that the substances are not one and the same, because they observed that the "selective absorbing ingredient" could be removed from culture filtrates with activated charcoal without significantly lowering the toxicity of the filtrate. Also the results with an ultrafiltered toxin showed that the ingredient, which is soluble in ether, was present in both the toxic residue and the non-toxic filtrate. This pigment showed absorption bands similar to those of some of the porphyrins and appeared to be produced or liberated by the diphtheria bacillus under conditions also favorable for toxin production. These studies were carried out with toxigenic and non-toxigenic strains; the various colony phases of the organism were not touched upon. In a somewhat broader study, Wheeler (1940) found porphyrins present in cultures of 10 non-virulent, non-toxigenic strains of *C. diphtheriae*, of 3 strains of *C. ulcerans*, 2 strains of *C. ovis* and 1 strain of *C. hoagii* but not in cultures of 3 strains of *C. xerosis* and 1 strain of *C. hofmanni*. These observations are in accord with those of Wadsworth, Crowe, and Smith, in that the conditions under which pigment and toxin production are observed in cultures of virulent, toxigenic diphtheria bacilli are similar to those under which non-virulent bacilli and related species of *Corynebacterium* synthesize porphyrins.

10. REDUCTION OF POTASSIUM TELLURITE

The cells of *C. diphtheriae* become colored by the reduced metal when the culture is grown in the presence of salts of tellurous acid (Klett, 1900). Conradi and Troch (1912) proposed a medium containing tellurite for the isolation of diphtheria bacilli, because on this medium the colonies assumed a characteristic black color. Since then numerous authors have proposed methods and media based on this phenomenon for the rapid isolation of diphtheria bacilli from mixed cultures. Baerthlein's (1913) observation that a large, moist colony type produced a black color when grown on Conradi's medium, which Conradi considered typical for *C. diphtheriae*, and a fine, translucent, bluish type of colony produced only a light brown color is the first suggestion of the variation in the color of diphtherial colonies on tellurite media being associated with colony form. It is upon the appearance of the diphtherial colonies on potassium tellurite-chocolate agar that Anderson and his co-workers based their classification and judged the pathogenicity of the microorganisms for human beings. We have already seen that virulence and toxigenicity of the cultures are not associated with colony form. On the other hand, pigmentation of the colonies on the special tellurite medium is a property which can apparently be correlated with the colony form.

In connection with the reduction of potassium tellurite by diphtheria bacilli, Manzullo in 1938 described a rapid diagnostic *in vivo* test for diphtheria. His method is to touch the exudate or pseudomembrane in the back of the throat with a swab which has been dipped in a 2 per cent solution of potassium tellurite. In the case of diphtheria, the area thus treated is supposed to turn black in 5 to 10 minutes. In the original series of 75 patients, Manzullo obtained no false positive results and only 7.4 per cent of the cases of diphtheria gave negative reactions to the immediate tellurite test. The experiences of other investigators with this test have differed from that of Manzullo very strikingly. Fox, Rhoads, and Lock (1939) applied the test to 27 patients with throat infections. All of them gave a negative tellurite test in spite of the fact that *C. diphtheriae* was isolated on Loeffler's

medium from 17 of the patients. There was one false positive reaction among 10 cases of non-diphtheritic infections. Tomlin (1939) found 22.7 per cent false positives. Tombleson and Campbell (1939), in a study of 200 patients, reported agreement between the tellurite test and the bacteriological diagnosis in 67.5 per cent, and with the clinical diagnosis in 77 per cent of the cases. Definite blackening occurred in specimens from 84.3 per cent of the cases which were finally diagnosed as diphtheria. Blackening also occurred in 46.8 per cent of faucial lesions caused by organisms other than the diphtheria bacillus. In a study of 277 swabs from 84 patients, Cooper, Peters, and Wiseman (1939) experienced 22.8 per cent false negative reactions in 57 bacteriologically proven cases of diphtheria, and 55 per cent false positives in 27 patients in which *C. diphtheriae* was never demonstrated. Four strains of *C. diphtheriae* were isolated from Loeffler's medium which failed to appear on the potassium tellurite plates. Tynan (1939) reported 13 per cent false negative and 47 per cent false positive reactions in his study of 75 unselected patients. Murray (1939) in a study of 62 individuals found that 36.6 per cent of the non-diphtheritic infections in addition to 84.3 per cent of the diphtheria patients gave a positive Manzullo test. Of those individuals giving a negative test 20 per cent were definite cases of diphtheria. Among the series of 200 cases studied by Woodcock (1939), 113 diphtheria patients gave a positive swab in 91 per cent, and a positive Manzullo test in only 85 per cent of the cases. Of 39 non-diphtheria patients, 95 per cent gave a negative swab whereas only 21 per cent gave a negative test; of 48 doubtful diphtheritic infections, 73 per cent gave positive swabs and only 48 per cent gave a positive Manzullo test.

The consensus among those who have investigated the Manzullo test is that it is unreliable and cannot replace the bacteriological methods in use at the present time. It is not unreasonable to expect that, if colonies composed of diphtheria bacilli show a variation in the reduction of potassium tellurite, the pseudomembranes in the throat, also composed of diphtheria bacilli, will show a variation in the reduction of the tellurite salt. In

addition, one cannot ignore the fact that blackening of tellurite is a reduction process that may under suitable conditions be carried out by agents other than the diphtheria bacillus.

Anderson, Happold, McLeod, and Thomson reported that organisms from type *mitis* colonies were partially inhibited upon subculturing to chocolate tellurite agar. Morton (1935b) observed the inhibition of growth on chocolate tellurite agar of the S colony form as well as type *mitis*; and more recently Cooper, Peters, and Wiseman (1939), and Perry and Petran (1939) have cited instances where diphtherial organisms were isolated from Loeffler's medium but not detected on tellurite medium. If it is true that type *mitis* represents the smooth phase and type *gravis* some phase of the culture nearer the rough phase, then, on the basis of the properties associated with smooth and rough colony phases as cited by Hadley in 1927, one would expect type *gravis* to be more resistant to inhibitory substances than type *mitis*.

11. CELL MORPHOLOGY

Many factors, both intrinsic and extrinsic, exert an influence upon the morphology of the bacterial cell. It was recognized early that there are many morphological forms of the true diphtheria bacillus; and Westbrook, Wilson, and McDaniel (1900) attempted to classify the various forms, a classification which occasionally is used even today. They knew, of course, that cultures of *C. diphtheriae* are composed, not of one, but of many morphological forms. Only those forms which predominated, or were present in fairly large numbers were recorded for the culture. Almost all of the forms listed in their classification could be observed in any one culture if sufficient time was spent in the examination. Since the advent of the single-cell technic, the conclusion prevails that the various morphological forms of the diphtheria bacillus are of no hereditary significance, as the subcultures of a single cell of any morphological form may show a mixture of many morphological forms. This fact was demonstrated by Powell (1923) and Crowell (1926) and has been confirmed by all who have cultured single cells of this organism.

The various morphological forms that have been reported for the diphtherial organism may be listed as follows:

(A) *Rod forms.* By far the most common morphological form of the diphtherial organism is the rod. This form, however, is subject to a great many variations. Some of the factors which bring about such variations will be mentioned shortly. As a working classification, and based upon the appearances of the microorganisms after being stained with Loeffler's methylene blue, Westbrook, Wilson, and McDaniel grouped the morphological forms into granular rods, barred rods and solid-staining rods. Granular-barred forms were reported later (Schultz, 1909). These various rod forms may be grouped into four major groups, viz.: (a) granular rods, (b) granular-barred rods, (c) barred rods, and (d) solid-staining rods.

The significance of these forms is still a disputed problem, although the subject has received much attention because of its practical relation to the matter of diagnosis. These forms are readily changeable from one to another as numerous workers have shown. Wherry (1917) advanced the hypothesis that there is a normal evolution beginning with the D^2 type or smaller and developing successively into the D^1 , C^1 , A^1 and sometimes the A type (Westbrook classification). Others have agreed with this suggestion.

(B) *Thread and branching forms.* Long thread forms showing true branching were observed early in smears from diphtheritic membranes and in cultures. Egg media, serum agar, and potato media appear to favor the production of branching forms although they are observed in cultures on ordinary agar. Grasset and Grasset (1930) have described thread forms that were produced by growing the organism in the presence of bile. Thread forms from cultures that were suggestive of approaching roughness were seen by Neisser (1932) and similar forms, definitely associated with the rough colony forms, were described by Hobby (1935).

(C) *Spheroidal forms.* Under certain conditions it appears that the morphology of the diphtherial organism may assume a spheroidal form (Grubb and Koser, 1934). The coccus-like

cells of *C. diphtheriae* seem to be of two types: (1) those produced temporarily by the environment and not stable; and (2) those which seem to be stabilized and to be the result of some intrinsic change within the organism, such as the C-forms of Kuhn and Sternberg (1931) and the coccoid form reported by Stone and Hobby (1934).

The cell morphology of the diphtheria bacillus is of great practical significance because many tentative diagnoses are based upon it. One must be familiar with the influences which the colony phase and the environment may have upon the shape of the bacilli. Many of the factors are listed below.

Relation to colony form. When it was apparent that certain cultural characteristics often could be associated with colony form, it was to be expected that in the diphtherial species, as in many other bacterial species, certain morphological forms would show correlation with colony form. In the case of mucoid (M) colonies, no encapsulated organisms have been demonstrated. According to Hobby (1935), the organisms are longer and more granular than those obtained from smooth colonies. Organisms from smooth (S) colonies show more uniform rod forms than those from SR colonies. Although the cells are rod-shaped and stain unevenly, their outlines are not distorted to the same extent as are those from cells in the SR colony form (Morton, 1940). In the intermediate (SR) colonies, the cells are larger and the shapes more bizarre than is the case for cells from the other colony types (Cowan, 1927; Morton, 1935b). Organisms from the rough (R) colonies are long and filamentous, often forming long, intertwining threads (Hobby, 1935). Grasset and Grasset (1930) did not mention the colony phase in their report on the filamentous forms; but one recognizes that the cultures described by Neisser (1932) as filamentous forms ("Fadendiphtheriebazillen") were approaching the rough phase.

There is little information relative to the morphology of the organisms in the small colony forms prior to the studies described by Morton, (1935b), who found that organisms in the dwarf (D) colonies are short and somewhat thick with some tendency towards swollen forms and uneven staining by methylene blue.

Usually the cells stain solidly with the gram and methylene blue stains. They exhibit the typical arrangements of cells so common among corynebacteria. Organisms in the G colonies are very short, slender rods, sometimes nearly spherical. They are variable towards Gram's stain and stain unevenly with methylene blue.

Different sections of the same colony were observed by Zarniko (1889) to show variations in morphology and size of the diphtheria bacilli. This may be due to the differences in age of the various bacilli within a given colony or it may be due to the differences in the immediate surroundings of the bacilli in different sections of the same colony; both factors, as we shall see, are capable of exerting an influence upon cell morphology.

Relation to age of culture. Even during the normal growth of diphtherial organisms under the most favorable conditions, there is a great variation in the morphology of the cells. Many changes in morphology occur during the first 15 hours of growth on Loeffler's medium (Denny, 1903). The young cultures show solid-staining forms, and as the cultures grow older these forms give rise to larger and unevenly staining rods, which often show clubbing.

According to Clark and Ruehl (1919) who studied 70 strains of different species at brief intervals up to 48 hours and then at longer intervals for one week, striking changes in morphology take place during the early hours of growth. In all instances, except in the case of the diphtherial group, the organisms found in young cultures, 4 to 9 hours old, are larger than the forms found in 20- to 24-hour cultures. In the case of the diphtherial group, the young organisms, 2 to 6 hours old, are definitely smaller and more solid-staining than the older forms. Most of the strains do not attain the original size of the organisms inoculated until they are 12 to 18 hours old. These observations were, in part, confirmed by Henrici (1928) on a bacillus belonging to the diphtherial group. On the other hand, Albert (1921), working with 125 pure cultures of the diphtheria bacillus, did not corroborate the findings of Clark and Ruehl. He observed a slight increase in size during the first 4 hours, maintenance of that size

for about 20 hours, and then a rapid diminution after the 24-hour period. At the end of 7 days, the average length was only about one-third of the maximum. In regard to shape, the young bacilli, according to Albert, are rather bluntly pointed; with the development of granules, the ends become more rounded, even bulging. With the disappearance of the granules during the process of disintegration, the bacilli become irregular in shape with the ends again more pointed. Young bacilli stain solidly; but after a few hours, the protoplasm of some of the bacilli in certain areas takes a heavier stain than the intermediate portions, thus producing a barred appearance. The metachromatic granules appear in cultures 4 to 8 hours old. They are small at first, attaining their largest average size in cultures 12 to 15 hours old. After this period, the granules diminish in size and disappear. The percentage of bacteria containing granules increases rapidly from the 4-hour period, when there are but few granules, to the 12-hour period when 91 per cent of the bacteria contain granules. At the end of two days most of the bacilli have lost their granules.

Powell (1923) studied 299 single-cell cultures and found that they pass through different morphological phases similar to those derived directly from colonies. After a 4-hour incubation period on Loeffler's medium, the cultures contain only solid forms similar to the C² forms of Wesbrook. After 8 hours the morphology is still the same. After 12 hours the solid-staining forms still predominate but granular forms similar to the C forms begin to appear. After 24 hours all the cultures show a predominance of granular forms of the C type. A, B, and other granular forms are also present and some of the cultures show barred forms as well. During the next 24 hours the morphological picture remains about the same, except for the appearance of large irregular forms with many of the bacilli staining less distinctly with methylene blue. The single-cell cultures present the same morphological picture as do the corresponding colony cultures.

Customarily, observers view diphtherial cultures after 18 to 36 hours of incubation. In 1934, Solé introduced a method for

the rapid culturing of diphtheria bacilli; and since the reports of Brahdý *et al.* (1934, 1935) it is coming into greater vogue. For this method, sterile cotton swabs are impregnated with sterile, undiluted, unheated horse serum without preservative and heated over a flame to coagulate the surface. After swabbing the nose or throat, the swab is placed in a sterile, dry tube, incubated 2 to 4 hours and smears made directly from the swab. The diagnosis of such smears quite obviously necessitates a familiarity with the morphology of diphtheria bacilli in cultures 2 to 4 hours old, rather than the classical morphological pictures based on cultures 18 to 36 hours old.

Relation to pH of the culture medium. In acid media (+2 per cent), Denny (1903) observed that the organisms after 48 hours' incubation are short and sometimes oval in shape with well-marked granules. Alkaline media (−2 per cent) do not have as much effect on the morphology of the diphtheria bacilli as does an acid medium. Bunker (1917), on the other hand found that very acid media (pH not given) yield large, irregular, solid-staining forms in pure culture, while very alkaline media give minute, solid-staining, triangular forms, resembling Wesbrook's D² forms. Between these extremes practically all of Wesbrook's forms can be observed. Laybourn (1921) reported that acid media (about pH 6.1) give small, irregular forms with few granules, and that alkaline media induce minute, triangular forms with few or no granules. The observations of Morton (1935b) confirmed in part those of Denny and of Laybourn in that acid media (about pH 6.2) favor the production of small forms. However, a systematic examination of the effect of pH on the cell morphology of the diphtheria bacillus has yet to be made.

Presence of glucose in the culture medium. Heinemann (1917) and Yarisawa (1926) noticed that the presence of glucose favors the production of coccoid forms. This effect may perhaps be associated with the acidification of the medium as a result of the fermentation of the glucose; but acidification is not the entire explanation. Morton (1935b) confirmed these observations and in addition reported that media with a pH corresponding to that in tubes in which glucose is being fermented give small forms but these are not coccoid.

Relation to oxygen tension. Wherry (1917) observed that when a culture of the diphtheria bacillus is grown aerobically on Loeffler's medium the A¹, C¹, and D¹ types of Wesbrook predominate, whereas when the culture is grown anaerobically, the D² type predominates. In confirmation, Schneider (1931) and Beck (1933) likewise observed that anaerobic growth yields short, thick, solid-staining forms devoid of polar granules. In addition to studying the effect of oxygen on the morphology of *C. diphtheriae*, Beck studied the influence of other gases, as well. He found that H₂, CO₂ and H₂S, although without effect on the virulence of the microorganisms, do bring about changes in the morphology. The presence of hydrogen sulfide in the atmosphere results in a temporary increase in the rate of growth and in the formation of granules.

Morphology in various media. Coccoid forms of the diphtheria bacillus have been observed to arise in tryptic digest broth prepared from horse muscle (Parish, 1927); these forms reverted, however, to rod forms in the first subculture on Loeffler's medium. Parker (1928), employing tryptic serum agar, has noticed a somewhat similar phenomenon. Forty-seven per cent of 57 diphtherial strains are reported by Grubb and Koser (1934) as promptly changing their morphology to coccus forms when grown on liver infusion medium. Eighteen-hour cultures on inspissated horse serum are described as yielding short bacillary forms, whereas agar yields long forms (Barratt, 1924). The presence of blood in the medium is said to favor the production of granules (Megraill, 1922). Bile and bile salts are unique in that their presence in culture media is said to produce long filamentous forms with numerous branches and many metachromatic granules (Berthelot, Ramon, Grasset, and Amoureux, 1927; Grasset and Grasset, 1930). The presence of CuSO₄ in the culture medium has been observed to change the morphology of diphtheria bacilli to that of cocci (Pope and Pinfield, 1932; Morton 1935b, 1940). Morton has observed, in addition, that MgSO₄, Na₂SO₄, Cu(NO₃)₂, MgCl₂, and (NH₄)₂SO₄ have somewhat the same effect.

The addition of guinea pig blood to agar favors the formation of coccoid forms (Yarisawa, 1926). Sterile emulsions of guinea

pig liver or kidney when added to agar have been noticed by Gins and Jermoljewa (1929) to change the morphology of diphtheria bacilli to that of the Hofmann bacillus. This change has also been observed by Schneider (1931) and Malcherek (1932). The first subcultures to Loeffler's medium from the peritoneal fluid of a guinea pig, that had been injected 24 hours previously by Crowell (1926) with a single-cell culture of diphtheria bacilli, have yielded a pure culture of coccoid forms. Subsequent transplants to Loeffler's medium from the culture of coccus forms have yielded pure cultures of rod forms. Maver (1931) has confirmed Hadley's (1907) observations that coccoid and solid-staining forms are produced when diphtheria bacilli are cultured in proteid-free media. Maver emphasizes that when there is a sudden change in the composition of the culture medium, the organism shows marked variations in morphology but that upon continued cultivation on the same medium the morphology gradually reverts to the more common rod forms. These observations may be the explanation for the change in morphology noticed when diphtherial microorganisms are cultured directly from the animal body. McGuigan and Frobisher (1936) recommend that the diagnosis of diphtheria should not be based upon the microscopical examination of smears made directly from colonies on tellurite medium, because the organisms often are small, thick, coccoid, and solid-staining. Involution forms have been reported as being not so abundant on amniotic fluid agar as on Loeffler's medium (Giltner and Ludlum, 1916).

Temperature employed for sterilization of Loeffler's medium. The amount of heat employed during the sterilization of the Loeffler's medium influences the morphology of diphtherial microorganisms when cultured upon it (Yarisawa, 1926; Morton, 1935b). The medium heated at 80°C. gives best coccoid formation, but when heated at 90°C. it yields typical rod forms. Perry and Petran (1939) also recognize the importance of the temperature of sterilization of Loeffler's medium in this connection.

Morphology in a mixed culture with other organisms. Smirnow (1908) in studying some symbiotic relations of *C. diphtheriae*

with other microorganisms has observed that such relationships give rise to coccoid forms. The change in morphology is not permanent as the diphtheria bacilli return to their typical morphology when grown under favorable conditions. Stovall, Scheid and Nichols (1923) have found that when a pure culture of *C. diphtheriae* is mixed and grown with a pure culture of *Staphylococcus aureus*, the diphtheria bacilli change their morphology from large club-shaped organisms with large granules to small thin organisms staining with heavy bands. No such effect is observed when non-hemolytic streptococci are substituted for the *Staphylococcus aureus* culture.

Discussion. From the relatively few citations of the voluminous literature it is evident that many factors, in the diphtherial organism itself, and in its environment, produce marked effects on the morphology of the cells. These factors have been cited because of their immediate practical significance.

In the study of the colony forms produced by a bacterial species, much information relative to the colony phase of the species often can be obtained by studying the morphology of the microorganisms. In the case of the diphtherial microorganism, however, many unusual circumstances are present. The genus *Corynebacterium* is exceptional in that during the normal growth and development of the microorganisms, the individuals are smaller during the first few hours of growth, then gradually attain their normal size. In the case of microorganisms of other genera the individual cells are slightly larger during the first few hours of growth. The so-called "normal" cells for the diphtherial species may be (1) rod forms, either granular, granular-barred, barred, or solid-staining; or (2) filamentous and branching forms; or (3) spheroidal forms. The significance of these various cell forms is not clearly understood at the present time. All conditions in the environment being as uniform as possible, the microorganisms in the R colony form are characterized by long, filamentous forms which stain solidly and often show branching. Microorganisms in the SR colony forms show a greater tendency towards bizarre forms; while those in the S colony form are more uniform in outline. The

organisms in the D colony form are characterized by their small size.

The diphtherial species is also outstanding in that the cell morphology is readily and markedly changed by the environment. The physiological youth and age of the culture, the reaction of the medium, the gaseous environment, the composition of the medium, and the temperature employed for sterilizing the medium have a decided influence upon the cell morphology. The sudden change of the microorganisms from one medium to another often results in a pronounced variation in the cell morphology. This variation is usually temporary, however, since continued subculturing upon one kind of medium results in restoring the microorganisms to their usual morphology. While many of the common factors that cause a temporary alteration in the cell morphology are known, there are doubtless many others still to be discovered.

SUMMARY

The diphtherial species, which in the modern conception of a bacterial species implies not only the diphtheria "bacillus" but all the cultural and morphological elements, is like many others in that it displays a variety of colony forms, namely, the mucoid (M), smooth (S), intermediate (SR), rough (R), dwarf (D), and gonidial (G) colony forms. The mucoid colony form, rarely reported, has not been demonstrated as yet to be made up of capsulated organisms or of organisms containing large amounts of a type-specific substance. The SR colony form, intermediate between S and R, occupies a greater rôle in the life history of the diphtherial species than similar forms usually occupy in other bacterial species. It is characterized by a pellicle type of growth in liquid media; such growth insures an adequate supply of oxygen to the growing organisms and thus enables them to develop more rapidly, and more readily to produce toxin or to ferment various carbohydrates. The R form is likewise a bit unusual in the diphtherial species in that the R colonies are smaller than the S, whereas in most other species it is the other way around. These various colony forms may be stable over a fairly long

period of cultivation or they may undergo spontaneous or forced variation. The trend in variation is from S to SR to R and reversion to the SR, or occasionally to the S form. Dwarf (D) colony forms have been observed to arise from the S, SR, and R forms. One is not able to predict with certainty what forms will arise when a given colony form is subjected to forced dissociation. In all cases, cultures from the S, SR, R, and D colony forms can be readily identified as the diphtherial species by their reactions towards the various test carbohydrates and specific immune serum, their virulence for guinea pigs, and their elaboration of the specific toxin.

Correlated with these various colonial manifestations is the manner of growth in liquid media. The M and S forms grow as uniform, homogeneous suspensions, the R form as a precipitate on the bottom of the vessel, and the SR form as a slight turbidity at first, which is replaced by a pellicle and sediment with the broth becoming clear. The D form grows as a very light turbidity and fine sediment, whereas the G form often fails to impart visible signs of growth in filtrates for a considerable period of time. When visible growth does occur, it is of a very fine turbidity with a ropy sediment. Suspended in physiological saline, microorganisms from the S colonies produce a uniform turbidity, those from the R colonies clump spontaneously, whereas organisms from the SR colonies give indeterminate results. Another property that appears to be correlated with colony form is the hemolytic activity of the cells. The S forms are hemolytic, the R forms less so, and the D forms non-hemolytic. Diphtherial cultures show the greatest hemolytic activity when 48 hours old; the hemolysin is thermolabile and non-filterable. Other members of the genus *Corynebacterium* have not been observed to possess this hemolytic property.

While the diphtherial microorganism reduces potassium tellurite, the various colony forms show differences in the rate of reduction and the degree of pigmentation. The differences in the amount of pigmentation of the colonies are probably due to the shape of the colony, the moisture content, and the texture of the surface. The S colonies (called "mitis" by the English

workers) which are raised, moist, and shiny appear black and shiny on tellurite medium. The SR colonies (called "gravis" by the English workers) are flatter and dryer, and appear grayish and dry on tellurite medium. The D forms which are slow in bringing about biochemical reactions are also slower than the S and SR forms in becoming pigmented.

Diphtherial microorganisms may be either virulent or avirulent for guinea pigs. If a strain is virulent, organisms from all the colony forms (S, SR, R, and D) may be virulent. There are, however, differences in the number of organisms required to produce death of the guinea pig in a stated time, the SR form having been found to produce death with fewer organisms than the S form. Quantitative tests have not been made with the R and D forms. In man, organisms from the S ("mitis") colony form appear to produce in the fauces a greater superficial membrane which is firm in texture and tends to extend into the intrathoracic air passages; and the most frequent cause of death is the respiratory obstruction produced by membrane formation. Organisms from the SR ("gravis") colony form appear to produce in the fauces less superficial membrane which is less firm in texture and shows less tendency to extend into the intrathoracic air passages; but this type produced deeper penetration of the tissues of the inflamed parts and greater involvement of the cervical lymph glands and surrounding tissues. In this case, the most frequent cause of death is the toxic effect on the viscera, especially the heart and kidneys. Toxigenicity does not appear to be the complete explanation for virulence, since the most virulent strains are not necessarily the greatest or most rapid toxin producers. Diphtherial strains may be either toxigenic or non-toxigenic. If toxigenic, organisms in all the colony forms (S, SR, R and D) are capable of elaborating the toxin. There are, however, differences in the rate and amount of toxin produced. The important question of why certain diphtherial strains are virulent and toxigenic and other strains are not is still unanswered. These attributes of the diphtherial microorganism are not associated with any particular colony form, nor with a particular antigenic component or serological type, nor with any

known biochemical activity. Studies into the chemical composition and respiration of diphtheria bacilli have only recently begun, and should contribute valuable information on many of the obscure aspects of the physiology of the organism.

Another property of the diphtherial microorganism not associated with any particular colony form is serological specificity. Preliminary observations have shown that microorganisms in the S, SR, R, and D colony forms behave similarly towards type-specific sera. The antigen responsible for type specificity among diphtheria bacilli has not as yet been identified, but the chemical studies already under way by the Chinese investigators should furnish this much-needed information.

The fermentation reactions of the diphtherial microorganism do not vary qualitatively with the different colony forms, but there are variations in the rates and the amounts of acid produced. *C. diphtheriae* produces acid from glucose and usually not from sucrose, but there are exceptions. In view of the rarity with which sucrose is utilized and the uncertainties attending the older recorded instances of such utilization, a careful reinvestigation of this property by modern methods would be highly desirable. Acid is usually produced from dextrin, starch and glycogen, the latter two substances, especially, being more frequently attacked by organisms in the SR colony form than in the other colony forms, as judged by the usual fermentation tests. The first, and thus far the only, respiration studies of the different colony forms indicate that organisms in the S ("mitis") and SR ("gravis") colony forms alike utilize glycogen, whereas the usual fermentation tests show that only the SR forms utilize it, *i.e.*, produce acid. This sort of discrepancy emphasizes again the need for careful quantitative metabolic studies as a basis for reliable interpretation and characterization.

The cell morphology of the diphtherial microorganism is quite variable. In addition to the usual variations in morphological forms which accompany changes in colony form, extensive morphological changes take place during the normal growth of the microorganisms, changes that are greatly modified by the reaction of the medium, the composition and temperature of steriliza-

tion of the medium, oxygen tension and symbiotic relations with other microorganisms. In addition to showing a variety of sizes and shapes, the microorganisms may appear as granular, granular-barred, barred or solid staining rods which may show filaments or branching forms. They may also appear in spheroidal form which, in certain cases, is transitory and dependent upon the environment, and in others is more permanent and independent of the environment.

This review of the many isolated observations of earlier investigators and of the results of more recent studies on the diphtherial organism emphasizes the necessity for viewing the diphtherial species in a manner quite different from that heretofore demanded by the older monomorphic concept of a bacterial species. *C. diphtheriae* is not typified exclusively by the "bacillus" of Klebs and Loeffler but by a considerable diversity of morphological and cultural elements that, taken collectively and perhaps in a certain sequence, make up the diphtherial species.

BIBLIOGRAPHY

- ALBERT, H. 1921 Variations in the morphology of the diphtheria bacillus due to age. *Abstracts Bact.*, **5**, 14-5.
- ANDERSON, J. S., COOPER, K. E., HAPPOLD, F. C., AND MCLEOD, J. W. 1933 Incidence and correlation with clinical severity of gravis, mitis, and intermediate types of diphtheria bacillus in a series of 500 cases at Leeds. *J. Path. Bact.*, **36**, 169-82.
- ANDERSON, J. S., HAPPOLD, F. C., MCLEOD, J. W., AND THOMSON, J. G. 1931 On the existence of two forms of diphtheria bacillus—*B. diphtheriae gravis* and *B. diphtheriae mitis*—and a new medium for their differentiation and for the bacteriological diagnosis of diphtheria. *J. Path. Bact.*, **34**, 667-81.
- ANDREWES, F. W., BULLOCH, W., DOUGLAS, S. R., DREYER, G., GARDNER, A. D., FILDES, P., LEDINGHAM, J. C. G., AND WOLF, C. G. L. 1923 *Diphtheria. Its bacteriology, pathology and immunology.* His Majesty's Stationery Office, London.
- ARKWRIGHT, J. A. 1921 Variation in bacteria in relation to agglutination both by salts and by specific serum. *J. Path. Bact.*, **24**, 36-60.
- BAERTHLEIN, K. 1913 Ueber Mutation bei Diphtherie. *Zentr. Bakt. Parasitenk.* I Ref., **57**, Beih., 89-92.
- BAERTHLEIN, K. 1918 Ueber bakterielle Variabilität, insbesondere sogenannte Bakterienmutationen. *Zentr. Bakt. Parasitenk.*, I Orig., **81**, 369-435.
- BARRATT, M. M. 1924-25 A study of *C. diphtheriae* and other members of the genus *Corynebacterium* with special reference to fermentative activity. *J. Hyg.*, **23**, 241-59.

- BECK, A. 1933-34 Der Einfluss der Anaerobiose und verschiedener Gase auf das Wachstum und die Virulenz von Diphtheriebazillen. Zentr. Bakt. Parasitenk., I Orig., **130**, 287-300.
- BELL, A. S. G. 1922 The serological differentiation of some strains of *Bacillus diphtheriae*. J. Roy. Army Med. Corps, **38**, 48-53.
- BERNHARDT, G. 1915 Über Variabilität pathogener Bakterien. Z. Hyg. Infektionskrankh., **79**, 179-248.
- BERNHARDT, G., AND PANETH 1913 Ueber die Variabilität des Diphtheriebazillus. Zentr. Bakt. Parasitenk., I Ref., **57**, Beih., 83-89.
- BERTHELOT, A., RAMON, G., GRASSET, E., AND AMOUREUX, G. 1927 Sur quelques particularités des cultures de bactéries et de champignons inférieurs en milieux biliés. Comp. rend. soc. biol., **96**, 963-5.
- BISSET, K. A. 1938 The structure of "rough" and "smooth" colonies. J. Path. Bact., **47**, 223-9.
- BRAHDY, M. B., BRODY, H., GAFFNEY, C. A., LENARSKY, M., AND SMITH, L. W. 1934-35 Comparison of a rapid (Folger-Solé) method and the routine Loeffler's method for diagnosis of diphtheria. Proc. Soc. Exptl. Biol. Med., **32**, 548-51.
- BRAHDY, M. B., LENARSKY, M., SMITH, L. W., AND GAFFNEY, C. A. 1935 A rapid method for the identification of diphtheria bacilli. J. Am. Med. Assoc., **104**, 1881-3.
- BRIEGER, L., AND FRAENKEL, C. 1890 Untersuchungen über Bakteriengifte. Berlin. klin. Wochschr., **27**, 241-6.
- BUNKER, J. W. M. 1917 Some effects of hydrogen ion concentration of media on the behavior of the diphtheria bacillus in culture. Abstracts Bact., **1**, 31-2.
- BUNKER, J. W. M. 1919 Studies of the diphtheria bacillus in culture. J. Bact., **4**, 379-407.
- CARTER, H. S. 1933 An analysis of 510 strains of *Corynebacterium diphtheriae*. J. Hyg., **33**, 542-6.
- CARY, W. E. 1917 Virulence and toxin-formation in *B. diphtheriae*. J. Infectious Diseases, **20**, 244-71.
- CHINN, B. D. 1936 Characteristics of small colony variants with special reference to *Shigella paradysenteriae*, Sonne. J. Infectious Diseases, **59**, 137-51.
- CHRISTISON, M. H. 1933 The stability of the mitis, intermediate and gravis types of *B. diphtheriae*. J. Path. Bact., **37**, 243-52.
- CLARK, P. F., AND RUEHL, W. H. 1919 Morphological changes during the growth of bacteria. J. Bact., **4**, 615-29.
- COBBETT, L., AND PHILLIPS, G. C. 1897 The pseudo-diphtheria bacillus. J. Path. Bact., **4**, 193-205.
- CONRADI, H., AND TROCH, P. 1912 Ein Verfahren zum Nachweis der Diphtheriebazillen. Münch. med. Wochschr., **59**, 1652-3.
- COOPER, K. E., PETERS, B. A., AND WISEMAN, J. 1939 Reduction of potassium tellurite in diphtheria and other throat conditions. Lancet, II, 248-50.
- COSTA, S., TROISIER, J., AND DAUVERGNE, J. 1918 Action hémotoxique du *B. diphtérique*. Sa valeur diagnostique. Comp. rend. soc. biol., **81**, 89-91.

- COULTER, C. B., AND STONE, F. M. 1930-31 The occurrence of porphyrins in cultures of *C. diphtheriae*. J. Gen. Physiol., **14**, 583-96.
- COWAN, M. L. 1927 The separation of virulent cultures of *B. diphtheriae* into virulent and avirulent types. Brit. J. Exptl. Path., **8**, 6-11.
- CROWELL, M. J. 1926 Morphological and physiological variations in the descendants of a single diphtheria bacillus. J. Bact., **11**, 65-74.
- DAWSON, M. H. 1928 The interconvertibility of "R" and "S" forms of pneumococcus. J. Exptl. Med., **47**, 577-91.
- DENNY, F. P. 1903 Observations on the morphology of *B. diphtheriae*, *B. pseudodiphtheriae*, and *B. xerosis*. J. Med. Research, **9**, 117-34.
- DUDLEY, S. F. 1925 The virulence of *Corynebacterium diphtheriae*, especially of the atoxic "subspecies". Brit. J. Exptl. Path., **6**, 225-34.
- DURAND, P. 1918 Classification des bacilles diphtériques par l'agglutination. Compt. rend. soc. biol., **81**, 1011-3.
- DURAND, P. 1920 Agglutination des bacilles diphtériques, Préparation des sérums—Techniques de l'agglutination et de l'absorption des agglutinines. Compt. rend. soc. biol., **83**, 611-3.
- DURAND, P. 1920 Les types de bacilles diphtériques déterminés par les épreuves d'agglutination et d'adsorption des agglutinines. Compt. rend. soc. biol., **83**, 613-5.
- DURAND, P. 1921 Action des bacilles diphtériques sur les hydrates de carbone. Compt. rend. soc. biol., **84**, 982-3.
- EAGLETON, A. J., AND BAXTER, E. M. 1923 The serological classification of *Bacillus diphtheriae*. J. Hyg., **22**, 107-22.
- EATON, M. D. 1934 Studies on pneumococcus variation. I. Variants characterized by rapid lysis and absence of normal growth under the routine method of cultivation. J. Bact., **27**, 271-91.
- EDWARDS, P. R. 1931 Studies on *Shigella equirulis* (*Bact. viscosum equi*). Kentucky Agri. Expt. Sta. Bull., #320, Sept.
- EIJKMAN, C. 1901 Ueber Enzyme bei Bakterien und Schimmelpilzen. Zentr. Bakt. Parasitenk., I Orig., **29**, 841-8.
- EISENBERG, P. 1914 Untersuchungen über die Variabilität der Bakterien. IV. Ueber den Variationskreis des *B. prodigiosum* und *B. violaceum*. Zentr. Bakt. Parasitenk., I Orig., **73**, 449-66.
- ERZIN, N. 1936 Untersuchungen über die Variabilität der Typen des Diphtherie-bazillus. Zentr. Bakt. Parasitenk., I Orig., **137**, 97-104.
- EWING, J. O. 1933 The serological grouping of the starch fermenting strains of *C. diphtheriae*. J. Path. Bact., **37**, 345-51.
- FITZGERALD, J. G., AND DOYLE, D. G. 1923 Agglutination test for the presence of *Bacillus diphtheriae* in field (mixed) cultures. J. Am. Med. Assoc., **80**, 1675-7.
- FLYNN, C. S., AND RETTGER, L. F. 1934 Variation and filtrability studies on *B. mesentericus* and *B. vulgatus*. J. Bact., **28**, 1-18.
- FOX, W. W., RHOADS, P. S., AND LACK, H. 1939 Experience with a rapid clinical test for diphtheria (Manzullo). J. Am. Med. Assoc., **113**, 675-6.
- FROBISHER, JR., M. 1939 Strains of *C. diphtheriae* in various parts of the United States. Am. J. Pub. Health, **30**, Supplement, 28-35.
- FROMME 1911 Ueber einen atypischen Typhusstamm. Zentr. Bakt. Parasitenk., I Orig., **58**, 445-53.

- FÜRTH, J. 1922 Variationsversuche mit Paratyphus β (Weil). Z. Immunitäts., **35**, 155-61.
- FÜRTH, J. 1922 Rezeptorenanalyse und Variationsversuche mit *B. paratyphus*, Aertryck. Z. Immunitäts., **35**, 162-75.
- GILTNER, W., AND LUDLUM, L. C. 1916 Amniotic fluid as a bacterial culture medium. J. Bact., **1**, 91-92.
- GINS, H. A., AND JERMOLJEWA, Z. 1929 Beiträge zur Morphologie und Biologie der Diphtheriebacillen. Z. Hyg. Infektionskrankh., **109**, 26-33.
- GOLDIE, H. 1933 Caractères hémolytiques des cultures de *Bacille diphthérique*. Compt. rend. soc. biol., **112**, 1210-12.
- GRASSET, E., AND GRASSET, G. 1930 Phase mycosique du cycle évolutif du *Corynebacterium diphtheriae*. Compt. rend. soc. biol., **103**, 1073-5.
- GRUBB, T. C., AND KOSER, S. A. 1934 Coccus forms of *C. diphtheriae*. J. Bact., **27**, 45.
- HADLEY, P. B. 1907 The growth and toxin production of *Bacillus diphtheriae* upon proteid-free media. J. Infectious Diseases, Sup. 3, 95-107.
- HADLEY, P. 1927 Microbic dissociation. J. Infectious Diseases, **40**, 1-312.
- HADLEY, P. 1937 Further advances in the study of microbic dissociation. J. Infectious Diseases, **60**, 129-92.
- HADLEY, P., DELVES, E., AND KLIMEK, J. 1931 The filterable forms of bacteria: I. A. filterable stage in the life history of the Shiga dysentery bacillus. J. Infectious Diseases, **48**, 1-159.
- HAMMERSCHMIDT, J. 1924 Ueber Gruppenbildung bei den Diphtheriebazillen. Zentr. Bakt. Parasitenk., I Orig., **93**, 443-59.
- HARTLEY, P. 1923 On the identity of the toxins produced by serologically different strains of *Bacillus diphtheriae*. Lancet, I, 17-18.
- HAUDUROY, P. 1927 Toxines diphthériques donnant naissance à un *Bacille diphthérimorphe*. Compt. rend., **184**, 409-11.
- HAUDUROY, P. 1927 Techniques de culture des formes filtrantes invisibles des microbes visibles. Compt. rend. soc. biol., **97**, 1392-4.
- HAVENS, L. C. 1920 Biologic studies of the diphtheria bacillus. J. Infectious Diseases, **26**, 388-401.
- HEEREN, R. H., AND MEGRAIL, E. 1930 The relation of hemolysins and toxins in diphtheria cultures isolated from acute cases. J. Infectious Diseases, **46**, 485-90.
- HEINEMANN, P. G. 1917 The morphology of a strain of *B. diphtheriae*. J. Bact., **2**, 361-3.
- HENRICI, A. T. 1928 Morphologic variation and the rate of growth of bacteria. C. C. Thomas, Springfield, Ill.
- HILL, H. W. 1903 Preliminary note on chromogenic cultures of *B. diphtheriae*. Science, **17**, 375.
- HOBBY, G. L. 1935 A study of variation in *Corynebacterium diphtheriae*. J. Infectious Diseases, **57**, 186-200.
- JONES, L. 1930-31 Virulence and electrophoresis of *Bacillus diphtheriae*. Proc. Soc. Exptl. Biol. Med., **28**, 883-4.
- KLEIN, E. 1890 Zur Aetiologie der Diphtherie. Zentr. Bakt. Parasitenk., I Orig., **7**, 489-93, 521-8.
- KLEIN, E. 1890 Ein weiterer Beitrag zur Aetiologie der Diphtherie. Zentr. Bakt. Parasitenk., I Orig., **7**, 785-94.

- KLEIN, E. 1890 Further report on the etiology of diphtheria. (Gt. Brit.) 19th Ann. Rept. Loc. Govt. Board, 143-214.
- KLETT, A. 1900 Zur Kenntniss der reducirenden Eigenschaften der Bakterien. Z. Hyg. Infektionskrankh., **33**, 137-60.
- KNOX, R., AND PASSMORE, R. 1938 Oxygen uptake of washed suspensions of *C. diphtheriae* in the presence of glucose and glycogen. J. Path. Bact., **46**, 303-8.
- KOPELOFF, N. 1934 Dissociation and filtration of *Lactobacillus acidophilus*. J. Infectious Diseases, **55**, 368-79.
- KOSER, S. A. 1929-30 On the production of variant colonies by certain of the intestinal bacteria. Proc. Soc. Exptl. Biol. Med., **27**, 975-7.
- KOSER, S. A., AND DIENST, R. B. 1934 Small colony variants or G forms of *Eberthella dysenteriae*, Sonne. J. Infectious Diseases, **54**, 131-47.
- KOSER, S. A., AND STYRON, N. C. 1930 Dissociation of *Bacterium dysenteriae*, Sonne, as influenced by variations in culture medium. J. Infectious Diseases, **47**, 453-77.
- KUHN, P., AND STERNBERG, K. 1931 Ueber Bakterien und Pettenkoferien. Zentr. Bakt. Parasitenk., I Orig., **121**, 113-61.
- LAYBOURN, R. L. 1921 The effect of the reaction of media upon the morphology of the diphtheria bacillus. Abstracts Bact., **5**, 14.
- LIPSTEIN, A. 1903 Ueber Immunisierung mit Diphtheriebacillen. Zentr. Bakt. Parasitenk., I Orig., **34**, 421-8.
- LOEFFLER, F. 1884 Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphtherie beim Menschen, bei der Taube und beim Kalbe. Mitt. kaiserl. Gesundh., **2**, 421-99.
- MALCHEREK, G. 1932 Ueber morphologische Veränderungen der Diphtheriebazillen auf Organemulsion—Agarnährböden. Zentr. Bakt. Parasitenk. I Orig., **125**, 65-72.
- MANZULLO, A. 1928 Nuevo método para el cultivo del *Corynebacterium diphtheriae* y nuevo método para el diagnostico de la difteria del hombre. Folia Biol., 365-71.
- MARTIN, L. 1898 Production de la toxine diphtérique. Ann. inst. Pasteur, **12**, 26-48.
- VON MAUNU 1914 Eine einfache Methode, die echten Diphtheriebacillen von Pseudodiphtheriebazillen kulturell zu unterscheiden. Münch. med. Wochschr., **61**, 702-3.
- MAVER, M. E. 1931 The attenuation of the diphtheria bacillus in synthetic medium. Observations on dissociation, antigenic relations and fermentative reactions. J. Infectious Diseases, **49**, 9-28.
- MCGUITAN, M. K., AND FROBISHER, Jr., M. 1936 Mediums for the study of diphtheria. J. Infectious Diseases, **59**, 22-9.
- MCLEOD, J. W., ORR, J. W., AND WOODCOCK, H. E. DE C. 1939 The morbid anatomy of gravis, intermedius and mitis diphtheria: Observations on a series of 51 post-mortem examinations. J. Path. Bact., **48**, 99-123.
- MEGRAIL, E. 1922 Factors influencing development of metachromatic granules in the diphtheria bacillus. J. Infectious Diseases, **31**, 393-401.
- MENTON, J. 1932 The different forms of *Corynebacterium diphtheriae*. J. Path. Bact., **35**, 651-2.

- MENTON, J., COOPER, T. V., DUKE, F. W., AND FUSSELL, W. H. 1933 The different types of *Corynebacterium diphtheriae*. J. Hyg., **33**, 414-20.
- MITTAG, G. 1937 Ueber den Nachweis von Diphtheriekolonien mit herabgesetztem Tellerspaltungsvermögen auf einer Tellurkochblutagarplatte mit Zystinzusatz. Zentr. Bakt. Parasitenk., I Orig., **138**, 426-31.
- MORTON, H. E. 1932 The production of the "G" type colonies of *C. diphtheriae*, Park No. 8 strain. Am. J. Path., **8**, 605.
- MORTON, H. E. 1935a Observations on the variation of the diphtheria bacillus. Am. J. Med. Sci., **190**, 142.
- MORTON, H. E. 1935b Observations on the life history of the diphtheria bacillus. Thesis, University of Michigan, Ann Arbor, Michigan.
- MORTON, H. E. 1940 *Corynebacterium diphtheriae*. II. Observations and dissociative studies. The potentialities of the species. J. Bact., **40**, 755-815.
- MURRAY, J. F. 1935a A note on the stability of the *gravis*, *mitis* and intermediate types of *C. diphtheriae*. Brit. J. Exptl. Path., **16**, 532-4.
- MURRAY, J. F. 1935b 166 cases of diphtheria correlated with the type of *B. diphtheriae* concerned. J. Path. Bact., **41**, 97-106.
- MURRAY, J. F. 1935c The serological relationships of 250 strains of *B. diphtheriae*. J. Path. Bact., **41**, 439-45.
- MURRAY, J. F. 1939 The Manzullo immediate tellurite test in diphtheria. S. African Med. J., **13**, 787-8.
- NEISSER, H. 1932 Ueber Fadendiphtheriebazillen. Zentr. Bakt. Parasitenk., I Orig., **124**, 503-16.
- OKELL, C. C. 1929-30 The relationship of virulent to avirulent diphtheria bacilli. J. Hyg., **29**, 309-12.
- OKELL, C. C., AND PARISH, H. J. 1926 The virulence testing of the diphtheria bacillus and its practical application. J. Hyg., **25**, 355-65.
- ORCUTT, M. L. 1923 Mutation among hog-cholera bacilli. J. Exptl. Med., **38**, 9-15.
- PARISH, H. J. 1927 Coccidial forms of the diphtheria bacillus. Brit. J. Exptl. Path., **8**, 162-6.
- PARISH, H. J., WHATLEY, E. E., AND O'BRIEN, R. A. 1932 *B. diphtheriae*, *gravis* and *mitis*. J. Path. Bact., **35**, 653, also Brit. Med. J., II, 915-7.
- PARK, W. H., WILLIAMS, A. W., AND MANN, A. G. 1922 Immunological studies on types of diphtheria bacilli. I. Agglutination characteristics. II. Protective value of standard monovalent antitoxin. J. Immunol., **7**, 243-52.
- PARKER, H. B. 1928 Rough and smooth strains of *Corynebacterium diphtheriae* on trypsin-serum-agar. Brit. J. Exptl. Path., **9**, 207-12.
- PASSMORE, R. 1938 The metabolism of washed suspensions of *C. hofmannii*. J. Path. Bact., **46**, 309-14.
- PAXSON, W. H., AND REDOWITZ, E. 1922 *Bacillus diphtheriae*. Immunological types; toxin-antitoxin relationship. J. Immunol., **7**, 69-79.
- PERRY, C. A., AND PETRAN, E. 1939 Routine laboratory examinations for *C. diphtheriae*. J. Lab. Clin. Med., **25**, 71-8.
- PETERS, E. A. 1897 Diphtheria and pseudo-diphtheria bacilli. J. Path. Bact., **4**, 181-92.

- POPE, C. G., AND PINFIELD, S. 1932 The production of coccal forms of *C. diphtheriae* in media containing copper. Brit. J. Exptl. Path., **13**, 60-5.
- POVITZKY, O. R., EISNER, M., AND JACKSON, E. 1933 Effectiveness of standard diphtheria antitoxin against all types of diphtheria infection. J. Infectious Diseases, **52**, 246-52.
- POWELL, H. M. 1923 A biological study of the diphtheria bacillus. I. The virulence of pure-line strains. Am. J. Hyg., **3**, 109-20.
- POWELL, H. M. 1923 A biological study of the diphtheria bacillus. II. Morphology of pure-line strains. Am. J. Hyg., **3**, 357-61.
- POWELL, H. M. 1923 A biological study of the diphtheria bacillus. III. The property of agglutination in pure-line strains derived from a common parent-cell. Am. J. Hyg., **3**, 362-9.
- PRIESTLEY, H. 1911-12 An attempt to differentiate the diphtheroid group of organisms. (Pathological section) Proc. Roy. Soc. Med., **5**, 46-61.
- PRZEWSKI, W. 1912 Beitrag zur Kenntnis der Morphologie und Biologie der Diphtherie- und Pseudodiphtheriebacillen. Zentr. Bakt. Parasitenk., I Orig., **65**, 5-22.
- RAVEN, C. 1934 Dissociation of the gonococcus. J. Infectious Diseases, **55**, 328-39.
- RETTGER, L. F., AND GILLESPIE, H. B. 1933 Bacterial variation with special reference to pleomorphism and filtrability. J. Bact., **26**, 289-318.
- RIEMSDIJK, M. 1914 Ueber die bakteriologische Diphtheriediagnose und die grosse Rolle, welche *Bacillus Hofmanni* dabei spielt. Zentr. Bakt. Parasitenk., I Orig., **75**, 229-64.
- ROBINSON, D. T. 1934 Further investigations on the gravis, mitis, and "intermediate" types of *C. diphtheriae*: Type stability. J. Path. Bact., **39**, 551-68.
- ROBINSON, D. T., AND PEENEY, A. L. P. 1936 The serological types amongst gravis strains of *C. diphtheriae* and their distribution. J. Path. Bact., **43**, 403-18.
- ROE, A. F. 1932 Anaerobic methods and the dissociation of *Bacillus welchii*. Thesis. Univ. of Michigan.
- ROE, A. F. 1934 Dissociation of *Cl. welchii*. J. Bact., **27**, 46-7.
- ROUX, E., AND YERSIN, A. 1888 Contribution a l'étude de le diphtérie. Ann. inst. Pasteur, **2**, 629-61; 1890, **4**, 385-426.
- SCHICK, B., AND ERSETTIG, H. 1903 Zur Frage der Variabilität der Diphtheriebazillen. Wien. klin. Wochschr., **16**, 993-5.
- SCHNEIDER, B. 1931 Versuche über den Einfluss wechselnder Lebensbedingungen auf die morphologische und biologische Veränderlichkeit von Diphtheriebazillen. Zentr. Bakt. Parasitenk., I Orig., **121**, 213-29.
- SCHULTZ, O. T. 1909 The proportion of granular and barred forms of *Bacillus diphtheriae* in throat cultures. J. Infectious Diseases, **6**, 610-15.
- SCHWONER, J. 1904 Ueber die hämolytische Wirkung des Loefflerschen Bacillus. Zentr. Bakt. Parasitenk., I Orig., **35**, 608-18.
- SCOTT, W. M. 1923 Agglutination reactions of diphtheria bacilli. (Gt. Brit.) Loc. Gov't. Board, Rept. Public Health Med. Subjs. No. 22, 40-66.
- SIA, R. H. P., AND HUANG, C. H. 1939 Serological classification of *C. diphtheriae*. Proc. Soc. Exptl. Biol. Med., **41**, 348-9.

- SMIRNOW, M. R. 1908 Some symbiotic relations of the *Bacillus diphtheriae*. J. Med. Research, **18**, 257-76.
- SMITH, G. H., AND JORDAN, E. F. 1930 Protobacterial forms of *B. diphtheriae*. J. Bact., **20**, 25-40.
- SMITH, J. 1923-24 A study of diphtheria bacilli, with special reference to their serological classification. J. Hyg., **22**, 1-5.
- SMITH, S. C. 1930 Nature of diphtheria toxin-antitoxin floccules. Lancet, I, 529.
- SOLÉ, A. 1934 Schnellkultur von Diphtheriebazillen. Wien. klin. Wochschr, **47**, 713-4.
- STONE, F. M., AND HOBBY, G. L. 1934 I. A coccoid form of *C. diphtheriae* susceptible to bacteriophage. J. Bact., **27**, 403-17.
- STOVALL, W. D., SCHEID, E., AND NICHOLS, M. S. 1923 The influence on the morphology and staining of *B. diphtheriae* by growth in mixed cultures with staphylococci and streptococci. Am. J. Pub. Health., **13**, 748-53.
- STUART, R. D. 1938 Starch-fermenting strains of *C. diphtheriae* in New Castle-on-Tyne: A serological and clinical investigation. J. Path. Bact., **46**, 173-80.
- SWINGLE, E. L. 1933-34 Studies on small colony variants of *Staphylococcus aureus*. Proc. Soc. Exptl. Biol. Med., **31**, 891-3.
- TOMBLESON, J. B. L., AND CAMPBELL, R. M. 1939 The immediate tellurite test in diphtheria. Brit. Med. J., I, 1275-7.
- TOMLIN, E. 1939 Potassium tellurite in the diagnosis of diphtheria. Brit. Med. J., **1**, 1273-5.
- TRUMPP, J. 1896 Diphtherie- oder Pseudodiphtherie-Bacillen im Emphysema. Zentr. Bakt. Parasitenk., I Orig., **20**, 721-5.
- TYNAN, J. G. 1939 Manzullo's tellurite test for diphtheria. Brit. Med. J., II, 786.
- WADSWORTH, A., CROWE, M. O'L., AND SMITH, L. A. 1935 The spectroscopic investigation of bacterial toxins: The absorption spectra of the products of *C. diphtheriae*. Brit. J. Exptl. Path., **16**, 201-17.
- WESBROOK, F. F., WILSON, L. B., AND MCDANIEL, O. 1900 Varieties of *Bacillus diphtheriae*. Trans. Assoc. Am. Physicians, **15**, 198-223.
- WESELOWA, W. 1914 Hämolytische Eigenschaften des Diphtherievaccins. Zentr. Bakt. Parasitenk., I Ref., **60**, 356.
- WESSELOW, W. S. 1914 Hämolytische Eigenschaften des *Bacillus diphtheriae*. (Charkowsky Medizinsky Jurnal., 1914, No. 1.), Centr. allgem. Path., **25**, 585.
- WHEELER, M. W. 1940 Porphyrin production by corynebacteria. J. Bact., **40**, 163.
- WHERRY, W. B. 1917 Influence of oxygen tension on morphologic variations in *B. diphtheriae*. J. Infectious Diseases, **21**, 47-51.
- WHITLEY, O. R. 1934 *Corynebacterium diphtheriae gravis* found in Maryland. J. Lab. Clin. Med., **19**, 943-4.
- WONG, S. C., AND T'UNG, T. 1938 Polysaccharides of *C. diphtheriae*. Proc. Soc. Exptl. Biol. Med., **39**, 422-3.
- WONG, S. C., AND T'UNG, T. 1939 Type-specific polysaccharides of *C. diphtheriae*. Proc. Soc. Exptl. Biol. Med., **41**, 160-2.

- WONG, S. C., AND T'UNG, T. 1939 Immunological studies on the cellular constituents of *C. diphtheriae*. Proc. Soc. Exptl. Biol. Med., **42**, 824-8.
- WONG, S. C., AND T'UNG, T. 1940 Further studies on type-specific protein of *Corynebacterium diphtheriae*. Proc. Soc. Exptl. Biol. Med., **43**, 749-53.
- WOODCOCK, H. E. DE C. 1939 Manzullo's tellurite test for diphtheria. Brit. Med. J., II, 830.
- WRIGHT, H. A., CHRISTISON, M. H., RANKIN, A. L. K., PEARSON, R. C. M., AND CUTHBERT, J. A. 1935 Further observations on the types of *Corynebacterium diphtheriae*. J. Path. Bact., **41**, 447-67.
- WRIGHT, H. A., AND RANKIN, A. L. K. 1932 Biological types of diphtheria bacillus and their clinical significance. Lancet, II, 884-7.
- YARISAWA, C. 1926 On the coccoid formation of *B. diphtheriae*. Japan Med. World, **6**, 35-9.
- YÜ, H. 1930 A study on the dissociation of the diphtheria bacillus. J. Bact., **20**, 107-20.
- ZARNIKO, C. 1889 Zur Kenntniss des Diphtheriebacillus. Zentr. Bakt. Parasitenk. I Orig., **6**, 153-62; 177-82; 224-8.
- ZUPNIK, L. 1897 Ueber Variabilität der Diphtheriebacillen. Berlin. klin. Wochschr., **34**, 1085-7.

CORYNEBACTERIUM DIPHTHERIAE

II. OBSERVATIONS AND DISSOCIATIVE STUDIES—THE POTENTIALITIES OF THE SPECIES¹

HARRY E. MORTON

*Department of Bacteriology, School of Medicine, University of Pennsylvania,
Philadelphia, Pennsylvania*

Received for publication May 29, 1940

In spite of the fact that the diphtheria bacillus is one of the few species of microorganisms that have been extensively studied in the laboratory, it becomes necessary occasionally (especially during a period in bacteriology characterized by the birth of new theories relating to the nature and organization of bacteria), to repeat certain old experiments, as well as to perform new ones, for the purpose of collecting data which may either prove or disprove the applicability of the new theories to an individual species.

Practically every aspect of the diphtheria bacillus has, at one time or another, been the subject of dispute. The very fact that there are so many reported instances of variation in this species should immediately suggest that this organism would be especially suitable for studies in dissociative variation. It is true that there have been performed during recent times many studies in forced dissociation; but sometime during the studies, if they are to be broad and thorough, one must take cognizance of the reported cases of variation (Morton), and attempt some sort of correlation, rather than carrying the dissociative studies along certain narrow lines with the result, perhaps, of producing only additional and still unexplained instances of variation.

¹ From the Hygienic Laboratory, University of Michigan, Ann Arbor, Michigan and the Department of Bacteriology, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

As a result of the recent studies on dissociative variation in many species and of the integrating concept of Hadley (1937), who has attempted to coördinate these observations, with the effect of producing a new picture of the nature of the bacterial individual and of the bacterial species, there has developed in modern bacteriology a tendency to depict bacterial species in a manner quite different from that heretofore demanded by the older monomorphic concept. The facts of pleomorphism and of the quite normal character of the chief dissociative culture phases, are now well established and fairly generally accepted, although the problem of the ontogenetic significance of these phases, as viewed by Hadley in his ontogenetic concept, can not receive a final answer at this time. It is with a tentative acceptance of the hypotheses indicated above that the present investigation of dissociative variation of the diphtheria species was undertaken and carried forward.

For some bacterial species there has been found a definite trend in the transformation process. In those species we are now able to correlate physical and chemical properties with such attributes as colony form. Instead of the existence of a chaotic state in the variation processes of these species, there can now be recognized a very orderly trend in the dissociation process. The thought therefore arises,—will the general scheme of dissociation of the diphtheria bacillus be the same as that for quite different species? Some workers have attempted to make such a general plan fit perfectly, with slight consideration for the special types of variations of *Corynebacterium diphtheriae*, already recorded in the literature (Morton).

The most fundamental new facts that one could contribute to our knowledge of the diphtheria bacillus would be those gleaned from a study of its life history. It is possible that a systematic study of its dissociative behavior would contribute much towards knowledge of this sort, rather than making the situation more complex. To date, no real comparative study has been made of the various culture phases of the diphtheria bacillus, in the light of the recent advances in bacteriology, especially along the lines of dissociative variation.

An extensive review of the literature has been made (Morton)

to ascertain the nature of the variations already reported for *C. diphtheriae*, since there was no previous attempt to interpret such variants from the standpoint of dissociation. As a result of this review, many instances have arisen in which, in the light of our present knowledge, it appears possible to correlate apparent discrepancies with colony form. These apparent correlations have been verified by studying both laboratory and freshly isolated strains. In some instances there has been employed a strain of *C. diphtheriae* derived from a single cell. There also have appeared variants which seemed to be independent of colony form, and this type of variation will also be considered. The plan has been to examine cultures of the diphtheria bacillus under conditions of "normal" cultivation, as well as subjected to various dissociative incitants; and to study any new forms which might result from such procedures.

Not always can a certain variation be produced at will. When a culture of the diphtheria bacillus is subjected to a particular dissociative incitant, perhaps a certain variant will occur; and in the course of study of this particular variant perhaps other variants will occur, and so on. After numerous investigations into various channels, it is well to stop and take inventory of the data in order to see what kind of composite picture the various parts make, with the aim of arriving at a better understanding of the nature of the diphtherial species—if not of the diphtheria "bacillus."

Anticipating somewhat the outcome of this investigation, it may be said that the results tend to confirm the concept of Hadley (1937) regarding the necessity of viewing in a new light the nature of bacterial species. The diphtherial species for example, is not typified exclusively by the "bacillus" of Klebs and Loeffler, but by a considerable diversity of morphological and cultural elements that, taken collectively, and perhaps (as Hadley has implied) in a certain sequence, make up the diphtherial *species-microphyte*.

MATERIALS

1. *Cultures*. Subcultures of the Park 8 strain were received from the Hygienic Laboratory, University of Michigan; Parke,

Davis and Company, Detroit, Michigan; Sharp and Dohme Laboratories, Glenolden, Pennsylvania and the Western Pennsylvania Hospital, Pittsburgh, Pennsylvania. A single-cell strain was derived from the Sharp and Dohme subculture of the Park 8 strain by the method described by Kahn (1929). Clinical strains were isolated from field cultures which had been submitted to the Department of Health, Detroit, Municipal Hospital, Philadelphia, and the Pennsylvania State Laboratory, Philadelphia. A few strains were also isolated from healthy "carriers." *Gravis* and *mitis* cultures were received from the National Drug Company, Philadelphia, and the New York City Department of Health. Some of the *gravis* and *mitis* cultures received as such were originally obtained from England.

Some of the clinical strains satisfied the criteria for the smooth colony type. As these studies were begun before the publication of Anderson, *et al.*, it is not known how those strains collected early in the studies appeared on potassium tellurite chocolate agar, but those strains collected more recently, when classified as smooth have appeared as *mitis* strains on the special medium. Those strains received as *mitis* strains either showed S colonies or a mixture of colony types in which S colonies could be found. All the Park 8 strains were intermediate between smooth and rough. One of the strains was rougher in appearance than the other three but it was not a typical R phase and so had to be classified as an intermediate. Those strains received as *gravis* strains showed colonies typical for such strains or a mixture of colony types in which *gravis*-type colonies could be found. The colonies appeared similar to the SR type colony. No strain was ever isolated from clinical sources which was as rough in appearance as R strains which were produced by forced dissociation.

From year to year some of the strains have shown variations in their colony form, exhibiting a mixture of colony types when plated. The stock cultures were maintained on blood agar slants, and in recent times have been overlayered with sterile paraffin oil (Morton and Pulaski, 1937) and transferred at intervals of six months to one year.

2. *Media*. Media prepared from a beef infusion base were

employed. In the beginning Bacto-peptone or Bacto-proteose peptone was employed but these have been discontinued in favor of Bacto-neopeptone or Parke, Davis & Co. bacteriologic peptone.

Infusion agar was prepared by adding two per cent agar to infusion broth.

Glycerol broth and glycerol agar were prepared by adding 5 per cent by volume of c. p. glycerol to filtered infusion broth or infusion agar.

Blood agar was prepared by adding 5 per cent by volume of sterile, defibrinated, normal rabbit or horse blood, aseptically, to infusion agar which had been melted previously and cooled to 45°C.

Tellurite chocolate agar was prepared as described in 1931 by Anderson, Happold, McLeod and Thomson or Bacto-tellurite blood solution and Bacto-dextrose proteose no. 3 agar was used.

Loeffler's medium was employed if sterilization was carried out in the autoclave. Intermittent sterilization in the inspissator was not trusted unless the medium was prepared by mixing the sterile ingredients under aseptic conditions and the inspissator used for coagulating the medium and not for sterilization. Hinkleman (1923) and Dupray (1924) have reported methods for sterilizing Loeffler's medium in the autoclave, but the methods were not found as satisfactory as that of maintaining constant pressure within the autoclave by means of compressed air during the heating and cooling of the autoclave.

Hiss serum water was prepared by adding six grams of Bacto-peptone or Parke, Davis and Company peptone to 900 ml. of tap water; the reaction was adjusted to pH 7.4 and the solution boiled vigorously for a few minutes. This procedure was done routinely, as certain batches of peptone coagulate serum upon mixing. The boiled peptone solution was restored to original volume and 300 ml. of beef or horse serum were added. Twenty-two milliliters of brom-cresol purple stock solution, 0.04 per cent, were added for the indicator, the medium filtered through filter paper and dispensed into sterile test tubes in 5 ml. amounts. Sterilization was carried out in the autoclave at about 105°C.

for 15 minutes and the medium incubated 48 hours to check sterility. This method of sterilization not only rendered the medium sterile but also destroyed any enzymes likely to be present in the serum which might hydrolyze certain carbohydrates and thus tend to false interpretations of the reactions of the cultures under test, as Goldsworthy, Still and Dumaresq (1938) have recently described. Sterile solutions of the various carbohydrates employed in the fermentation tests were prepared by dissolving either 5 or 10 grams (depending on the solubility of the carbohydrate in water) of the pure carbohydrate in 100 ml. of distilled water and filtering through a sterile Berkefeld "N" or sintered glass filter (Morton and Czarnetzky, 1937). The sterile filtrates were then dispensed into the tubes of sterile Hiss serum water in the amounts of 0.5 ml., if the concentration of the carbohydrate was 10 per cent, or 1 ml., if the concentration was 0.5 per cent. This gave a final concentration of the carbohydrate in the Hiss serum water of approximately one per cent. Extract broth has been found to be a better basic medium in the fermentation test if the strain is not too fastidious.

Before using, media which had been stored in the cold room or refrigerator, were warmed to room temperature.

EXPERIMENTAL

1. *Influence of the reaction of the medium on the growth of the diphtheria bacillus.* Realizing that certain ranges in pH of the culture media are effective in bringing about, or stabilizing, certain colony types, one of the first things determined was the range in pH permitting growth of the diphtheria bacillus. The experiment was carried out by seeding one standard 4 mm. loopful of a 24-hour glycerol broth culture of the Park 8 strain, (Sr phase) into each of a series of tubes of glycerol broth of varying pH. The pH of the glycerol broth was carefully determined for each lot by means of the Leeds and Northrup quinhydrone pH indicator. The inoculated tubes of glycerol broth were incubated at 37°C. The results of the experiment are listed in table 1.

Practically the same results were obtained with a Park 8 strain from another source.

These results are comparable with those of Bunker (1919), who found that the diphtheria bacillus grew in a range of pH 5.7 to 8.7, and Dernby and David (1921), who found the range to be between pH 6.0 and 8.3 with the optimum between pH 7.2 and 7.8.

As a check on the macroscopic observations, glycerol agar plates were inoculated with three drops from the pH 5.6, 5.7, 6.2 and 8.5 tubes, respectively. The plate inoculated from the pH 8.5 tube showed no growth, whatsoever. The plate inoculated from the pH 6.2 tube showed numerous Sr colonies, which were typical of the strain when it was used as the inoculum for the

TABLE 1

Range in pH at which growth of C. diphtheriae, Park 8 strain took place

INCUBATION	pH 5.6	pH 5.7	pH 6.2	pH 6.6	pH 7.3	pH 7.7	pH 8.1	pH 8.5	pH 8.8
<i>hours</i>									
16	—	—	—	FC	C	—	—	—	—
24	—	—	—	FC	C	—	—	—	—
48	—	—	S, P	S, P	C, P	C, P	C, P	—	—
65	—	—	S, P	S, P	S, P	S, P	S, P	—	—
115	—	P*	S, P	S, P	S, P	S FC P	S, C P*	—	—

—, no visible macroscopic growth; FC, faint clouding; C, clouding; S, sediment; P, pellicle; * very small pellicle.

series of broth tubes. The plate inoculated from the pH 5.7 tube showed numerous very small colonies and only three large, typical colonies. The pH 5.6 tube showed only numerous very small colonies. Thus, small colony forms were encountered in the very first experiment in these studies. These particular small colonies will be described in detail in a later section.

2. *Serial transfer in alkaline glycerol broth.* It is generally known among bacteriologists interested in dissociation, that an alkaline medium usually favors the production of the rough culture phase. Therefore, continual cultivation of a strain in a favorable medium of the highest pH permitting growth is one of the commonest methods of producing transformation of a

strain to the rough phase. From the information obtained from the experiment in the immediately preceding section, serial transfers at 48-hour intervals were made of the Park 8 strain in tubes of glycerol infusion broth, pH 8.1. The serial transfers were carried on for 11 culture generations without evidence of change in the manner of growth in the broth or on glycerol agar plates. As the strain became adapted to the alkaline environment, more alkali was added to the tubes of glycerol broth in the form of sterile N/10 NaOH solution. By the twentieth culture generation, the strain was growing in the pH 8.1 glycerol infusion broth to which had been added 10 drops of sterile N/10 NaOH solution. Growth from this highly alkaline tube showed only the typical colonies which were present at the beginning of the series. The growth in the last tube in the series was allowed to evaporate to a resinous mass, over a period of 42 days, resuspended in sterile distilled water to the original volume of the broth and filtered through a sterile Berkefeld "N" filter under 34 cm. of negative pressure. At no time during the three months which elapsed did this filtrate give rise to growth on glycerol agar plates when treated by the Hauduroy (1927), technic, although positive results were obtained with other filtrates as will be described later.

3. *Serial transfer in acid glycerol broth.* Since serial transfer of the Park 8 strain in alkaline broth resulted in no change in the culture towards the R type, it was believed that serial transfer of the strain in acid glycerol broth might result in a purer S type culture. Serial transfer at 48-hour intervals of the Park 8 strain in glycerol broth of pH 6.2 for 23 culture generations resulted in no colonies which were smoother in appearance than those produced by the strain under ordinary conditions. Small colonies were encountered, however, which will be described in a later section.

4. *Heating at 39.5°C.* Roux and Yersin (1890) reported depriving a virulent diphtheria culture of its virulence by heating it at 39.5°C. for 25 days. Hewlett and Knight (1897) observed that heating at 45°C. for 24 hours killed all of the bacilli. Heating at 45°C. for 17 hours killed nearly all of the organisms; those that did live were completely changed "appearing like pseudo-

diphtheria bacilli." The organisms could be changed back to typical diphtheria bacilli by subculturing on serum at 37°C. for several generations.

Since it was found impossible to grow the Park 8 strain serially at a temperature of 43°C., the temperature of the incubator was lowered to 40°C. Throughout the experiment the temperature varied between 39.5 and 40°C. The strain was carried through 27 serial culture generations in glycerol infusion broth, covering a period of 32 days. The cultures and media were kept in the incubator except for about two minutes when the actual transfers to fresh media were made. There was no change in the manner of growth of the organisms in broth or on glycerol agar throughout the series. One and eight-tenths milliliters of the 24-hour culture from the twenty-seventh tube in the series was inoculated into a 280-gram guinea pig. As a control 2 ml. of a 24-hour broth culture of the parent strain grown at 37°C. was injected into a second guinea pig. Both animals were dead at the end of 40 hours. Typical diphtheritic post-mortem lesions were found.

Two milliliters of the culture from the first tube in the series, which had been kept at about 40°C. for the entire 32 days, proved to be non-pathogenic for a 315 gram guinea pig. This culture also failed to grow upon sub-culturing, which might explain the results obtained by Roux and Yersin.

5. *Effect of aging.* In a series of 25 broth cultures of the Park 8 strain contained in test tubes which were sealed as ampoules and allowed to age for 140 days at room temperature and in the dark, two of the ampoules (096 and 097) gave a smooth type of colony in addition to the typical SR colony of the Park 8 strain when the contents of the ampoules were streaked onto glycerol agar plates. The new, smooth colonies were round, convex, moist, smooth and shiny, with even margins. These smooth colonies were transferred to blood agar slants and grown in pure culture. They gave fermentation reactions typical for *C. diphtheriae*, were agglutinated by agglutinating serum prepared against the parent strain, but the cultures were not, at first, pathogenic for 250 gram guinea pigs. The strains were carried in stock as 096 and 097, respectively.

Numerous cultures of the diphtheria bacillus on solid media,

as they aged for weeks and months, were observed to show rough outgrowths from the margins of the intermediate colonies. No special attempt was made to isolate a rough strain by selectively culturing the rough-appearing secondary growth.

A series of 12 broth cultures of the Park 8 strain, Sr phase, were allowed to age at room temperature and in the dark. From time to time sterile distilled water was added to each tube to restore that lost by evaporation. Periodically, infusion agar plates were inoculated from the tubes. Only one of the 12 cultures eventually yielded rough variants. The same tube also produced the extremely small colony type.

6. *Growth of the Park 8 strain in the presence of antitoxin.* Several workers have reported that the growth of a toxigenic strain of the diphtheria bacillus in the presence of antitoxin has resulted in the strain becoming non-toxic. To determine whether or not such a change would be accompanied by any other change in the organisms, the Park 8 strain was grown serially in the presence of antitoxin. The diphtheria antitoxic horse serum was obtained from a diphtheria antitoxin-producing horse which was showing a potency of 900 units per milliliter of serum. The first 7 tubes in the series contained 10 per cent antitoxic serum in glycerol infusion broth. The next 5 tubes contained 20 per cent serum; the next two tubes, 50 per cent serum, the next tube, 75 per cent serum and the last three tubes in the series contained the undiluted antitoxic serum. In all, the strain passed through 18 culture generations in the presence of antitoxic antibodies. At no time did the strain show any variation in its colony form. A tube of plain glycerol broth was inoculated from the last tube in the series and incubated for 48 hours, the purpose of this tube being to eliminate the possibility of any antitoxin being transferred along with the inoculum when the strain was tested for toxin production. A flask of Bacto-proteose peptone infusion broth was inoculated from the 48-hour glycerol broth culture and, for a control, a second and similar flask of proteose peptone infusion broth was inoculated from a glycerol broth culture of the parent Park 8 strain maintained on Loeffler's medium. Both flasks were incubated at 37°C. for eight days and both showed the

characteristic pellicle and sediment with no clouding of the broth. Both cultures were filtered through a sterile Chamberland L3 filter under a negative pressure of not more than 5 cm. mercury. To a portion of each filtrate was added tricresol to the amount of 0.3 per cent and the toxins placed in the cold room. After aging in the cold room for two weeks, the toxins were injected in varying amounts into guinea pigs with the result that both toxins were of practically the same potency, having an M.L.D. of 0.02 ml.

From this experiment it was concluded (1) that the toxigenic Park 8 strain of *C. diphtheriae* does not necessarily lose its toxigenicity when grown in the presence of antitoxin; (2) that the Sr colony type of the Park 8 strain is not markedly changed by the growth of the organisms in the presence of antitoxic antibodies; and (3) that these observations corroborate the findings of Schick and Ersettig and of Yü, but are contrary to the findings of Bernhardt (1914), Becker, (1927) and Jungeblut (1927).

7. *Growth of the Park 8 strain in the presence of anti-bacterial serum.* The Park 8 strain was grown for 21 culture generations in the presence of its homologous immune serum without any change, whatsoever, occurring in the manner of growth of the strain in liquid or on solid media. Since the serum did not show complete agglutination in dilutions of 1:100, another attempt was made by growing the strain for 16 culture generations in the presence of a serum showing complete agglutination in a dilution of 1:1280. No change in the character of the strain was produced. Another serum showing partial agglutination in a dilution of 1:4,000 did not change the character of the strain after growing the organisms in the undiluted serum for eight culture generations. Finally a serum showing definite agglutination in a dilution of 1:10,240 was used against a Park 8 strain from another source. The organisms were grown for 24 culture generations in broth containing 50 per cent immune serum with no change in the appearances of the strain.

8. *The effect of growing the diphtheria bacillus in the presence of lithium chloride.* a. *Park 8 strain.* Growth of the Park 8 strain in glycerol infusion broth containing 0.5 per cent or more

of lithium chloride resulted in the production of small colonies, which will be described in a later section. No rough colonies were encountered with this strain.

*b. A freshly isolated strain.*² Since the Park 8 strain had failed to produce the rough colony form by the usual methods employed to produce the rough forms of other organisms, it was thought that perhaps a strain of the diphtheria bacillus freshly isolated from a patient might be more easily dissociated.

For the experiment a strain isolated from a field culture, definitely identified as a true diphtheria bacillus and carried in stock as *C. diphtheriae*, II, was employed. On plain agar it gave the smooth type of colony. This strain was inoculated into tubes of infusion broth containing one per cent LiCl plus one drop of sterile 50 per cent LiCl solution. Upon inoculating a one per cent LiCl agar plate from the second tube in this series, rough-appearing colonies were observed. The roughest colony was spread over the surface of another LiCl agar plate and the roughest colony on this plate transferred to a third LiCl agar plate, on which the colonies were uniformly rough. One of the roughest colonies was then transferred to a tube of 1 per cent LiCl broth. The growth from this tube was plated onto infusion agar and the roughest colony transferred from this plate to another tube of 1 per cent LiCl broth. This process was repeated for three more culture generations. It was thought that the selection of the roughest colony on each plate to serve as the inoculum for the broth tubes would hasten the change of the colony form of the strain to the extreme rough type. The roughest colony on the last plate in the series was again picked from the plate to another plate of LiCl agar and this showed all rough colonies. Again one of the roughest colonies was picked to another LiCl agar plate and this also showed all rough colonies. This demonstrated that the strain was producing only rough colonies. A rough colony was picked off the plate to a tube of one per cent LiCl broth plus two drops of sterile 50 per cent LiCl solution.

² I am indebted to Dr. Arthur Bernstein for his assistance in the production and the study of the rough colony variants.

The organisms grew as a granular sediment on the bottom of the tube. The strain was carried serially through 5 more tubes of 1 per cent LiCl broth each containing two drops of sterile 50 per cent LiCl solution. (The strain would not grow in the presence of more LiCl.) Upon plating out the growth in this tube, both smooth and rough colonies appeared. A rough colony was picked from this plate and carried serially through 8 more tubes of 1 per cent LiCl broth. The strain now showed upon plating, only extremely rough colonies. A glycerol agar plate and an infusion agar plate were inoculated from the same LiCl broth tube in this series. The infusion agar plate showed very rough colonies, whereas the colonies on the glycerol agar plate were not nearly as rough in their appearance. Similar parallel platings on glycerol agar and infusion agar throughout the experiment confirmed this observation and the observations of Zupnik (1897) and Schick and Ersettig (1903) that glycerol agar does not satisfactorily differentiate the rough and smooth colony types of *C. diphtheriae*. A guinea pig inoculated with all of the 24-hour growth of the rough culture from a blood agar slant died in 24 hours.

Believing that the culture was again fairly well established in the rough colony form, it was inoculated into plain infusion broth. Upon plating, this culture yielded only very rough colonies, one of which was fished to a tube of infusion broth. Upon plating this culture, only very rough colonies were obtained. The strain was passed through 4 more culture generations in infusion media and then inoculated to a blood agar slant and kept over the summer. In the fall this three months' old culture was carried through a series of four 1 per cent LiCl broth tubes. The first tubes in the series showed only rough colonies but the fourth tube showed 40 large smooth colonies scattered over the plate, which also contained small, extremely rough colonies, too numerous to count. A rough colony was then transferred to a tube of 1 per cent LiCl broth, the culture plated out and the roughest colony on the plate reseeded into a 1 per cent LiCl broth tube. This process was carried on for 9 culture genera-

tions. All of the cultures showed only the very rough, small colonies. A rough colony was transferred to a plain agar slant. This slant, after incubation, showed two raised, round, creamy, smooth colonies among the countless flat, irregular, bluish, rough colonies. A smooth colony was selected and grown in a pure culture, which was the third instance in which the colony type reverted to the smooth type after the strain had shown only rough colonies for several generations. The typical rough colonies were transferred to infusion agar slants and when plated on infusion agar plates yielded only extremely rough colonies. A culture of the colony pictured in plate I was propagated as the II R strain.

Many of the plates made from LiCl broth cultures showed numerous extremely small colonies, 0.2 mm. or less in diameter. Nothing was done with these, as they had been studied on other occasions and we were seeking the extreme rough colony phase.

In summary, it may be stated that the smooth, intermediate and rough colony types have been observed or produced in the following instances.

The smooth (S) colony type. The pure S has not been observed to be as common as the intermediate colony types in which the S characteristics predominate. The S colony type has been observed in field cultures, has been produced from the intermediate colony type by allowing broth cultures of the latter to age or by growing in media containing LiCl, and has been observed to arise spontaneously in cultures of the extreme R phase and by growing the latter in the presence of LiCl.

The intermediate colony type. Representatives of this group of intermediate colony forms were usually found to be present in strains of the diphtheria bacillus maintained on artificial culture media for a long time; it predominated in strains isolated from patients; it occasionally developed spontaneously from the rough colony type and also by culturing the rough type in the presence of LiCl. It might appear a bit unusual for one of the intermediate colony types to arise from the extreme R phase but this has been observed repeatedly, especially in stock cul-

tures of the R phase. Unless cultures in the R phase are maintained on solid media or occasionally grown in the presence of LiCl, or preserved under paraffin oil, or in the desiccated state, the very flat, bluish, irregular, granular colonies become thicker, more opaque, less irregular and show a central papilla.

The extreme rough (R) colony type. The extreme rough colony type was produced from the Sr type by aging broth cultures of the latter or by growing in the presence of LiCl.

APPEARANCE OF THE S, INTERMEDIATE AND R COLONY TYPES

Infusion agar. The S type colony is round and convex, with even margin and smooth surface. It is opaque when viewed by transmitted light, glistening and somewhat moist in appearance when viewed by reflected light. The growth is rather conservative on solid media as compared with the amount of growth produced by the intermediate type colonies. The colonies are usually about 1-3 mm. in diameter (plate I).

The intermediate type colonies comprise types intermediate between the pure smooth and rough phases, and have been designated by the symbols Sr, SR, and sR. In the transformation of S to R, the colony type slightly removed from the S phase has been referred to as the Sr type. The individual colonies are very similar to the S type colonies except that the surface is less convex, slightly drier, sometimes slightly pitted, and the margins may be slightly uneven and show a tendency to spread as the colonies age. The SR type colonies are distinctly larger than the S or R types, usually 3-5 mm. in diameter. By transmitted light the SR type colonies are opaque and when viewed by reflected light the surface is granular, not glistening, and drier than in the case of the S type colony. The surface is raised but not convex as in the case of the S type colony. Sometimes the surface is nearly level, at other times the central portion of the colony is raised and at other times there may be a central cone surrounded by a concentric depression and elevation. The margins usually show indentations. The growth is more luxuriant than with the other colony types and usually

shows a tendency to spread upon aging. The sR type colonies are thinner, the surface flatter and more uneven, the margins much more irregular and the colonies show a tendency to spread. The colonies very often show an opaque center which tapers off to a translucent margin, when viewed by transmitted light.

The extreme R type colony is smaller than the intermediate types and is usually slightly smaller than the S type, being about 1-3 mm. in diameter. The colony is flat and the margin very irregular. By transmitted light the colony is translucent; by reflected light the surface is pitted, very uneven and reflects little light. It is so flat and uneven that there was often insufficient contrast between it and the surface of the agar to permit photographing by reflected light.

Great difficulties may be encountered when describing colony forms if strict attention is not paid to the composition of the media. When comparisons were made, the various cultures were streaked upon plates poured from the same lot of media, prepared, incubated and examined under the same conditions and at the same time.

Colonies of the diphtheria bacillus, more than those of any other species of microorganism studied, varied greatly with the brand of peptone employed in the medium. Certain peptones, such as Bacto-neopeptone, Bacto-peptone, Bacto-proteose peptone, Chaissang's peptone, Medo-peptone, and Parke, Davis and Company's bacteriologic peptone, tend to produce moist colonies, some more so than others. Bacto-protone, Bacto-tryptone, Baker's peptone, Fairchild's peptone, Merck's peptone, Stearn's peptone and Witte's peptone yielded colonies very different from the ones obtained with the above mentioned peptones—some colonies so different that they would not have been judged diphtheria colonies by macroscopic examination had one not known the source of the culture. Some microorganisms grew nearly as well on plain extract agar without peptone as on the extract agar containing certain brands of peptone.

We have attempted to keep the composition of the culture media as simple as possible and still obtain good growth of the

diphtheria bacilli. Infusion agar containing Bacto-peptone or Parke, Davis and Company's peptone was used when it was necessary to have a clear medium for viewing the colonies by transmitted light. Infusion agar containing 5-10 per cent fresh, defibrinated rabbit blood was used for maintaining the cultures in stock and whenever a medium richer than ordinary infusion agar was required. Colony types can be distinguished as well on blood agar as on infusion agar. Indeed, Wilson and Goldsworthy have recently called attention to the fact that the *mitis*, *gravis* and intermediate colony types of the English workers can be distinguished very readily on infusion agar containing Parke, Davis and Company's peptone and 5 per cent guinea pig or rabbit blood. There is no question as to the colony types when one views their excellent photographs of the colonies on blood agar. The kind of blood, however, is very important in differentiation of colony type, as Knox (1937) has shown. Dudley (1934) stated that trypsin serum agar gave a clearer differentiation of colony types than did the potassium tellurite chocolate agar. Glycerol agar has been used a great deal but is not favorable for the differentiation of colony form.

Potassium tellurite chocolate agar. When grown on this special medium the S type colonies appeared very much the same as when grown on plain infusion agar except that the color of the colonies was black. The appearance of the S and *mitis* colonies were the same. Another striking similarity between the S and *mitis* cultures was that both types were inhibited by the potassium tellurite. Many strains failed to grow when transferred to this medium; other strains grew poorly.

The intermediate type colonies were very similar in appearance to those grown on plain agar except for the color of the colony. As the colony form approached the R phase, the color of the colonies became grayish. The grayish appearance of the SR colonies was perhaps accounted for by the fact that the surface of the colonies was pitted, drier and more uneven than in the case of the S type colonies which were smooth and moist. The SR colonies and *gravis* colonies had the same general appearance

on the tellurite medium. Another point in which the two types of colonies were similar was that they were not inhibited by the potassium tellurite, both growing luxuriantly.

The R type colonies were grayish, the color becoming more intense, sometimes, as the colonies aged.

TEXTURE OF SMOOTH, INTERMEDIATE AND ROUGH TYPE COLONIES

When a platinum needle was drawn through the S type colony, the colony, being moist, flowed over the needle, but the track of the needle did not disappear completely as when the needle is drawn, for example, through a drop of water. If the needle was drawn through an SR type colony, the colony appeared drier because it did not flow over the needle. Portions of the colony adhering to the needle did so as masses and did not appear to lose their shape or wet the needle. The needle left a distinct groove, or furrow, which did not tend to close. In the case of the R type, the colony was very dry and brittle. When the needle was drawn through an R colony, a section of the colony often broke off from the colony and moved along in front of the needle. Portions of the colony were picked up by the needle as particles which were hard to break up into finer particles.

APPEARANCE OF THE GROWTH IN LIQUID MEDIA

In these studies it has been observed that the S type colony produces a uniform turbidity in liquid media.

The intermediate type colonies, if near the S phase, Sr, produce both turbidity and sediment which is finely granular in contrast to the even turbidity produced by the S type. As the intermediate stage approaches the R phase the granules become coarser and give rise earlier to a sediment, very little turbidity being produced. The SR type produces an initial clouding of the broth which gives rise to a pellicle and sediment.

The extreme R type colony grows only as a very coarse, granular sediment.

Very similar to the appearance in broth was the appearance of the organisms of the various colony types when suspended in physiological salt solution. The S type produced uniform,

stable suspensions, the SR and R types granular, unstable suspensions.

PRODUCTION AND DESCRIPTION OF THE D (DWARF) COLONY TYPE

Fourteen test tubes and four 125 ml. Erlenmeyer flasks containing infusion broth, pH 7.2, were inoculated with a Park 8 strain, whose colonies were of the intermediate type, and after incubating 48 hours to insure good growth, the cultures were placed at room temperature and in the dark. Naturally the cultures dried out as they aged so that sterile distilled water had to be added aseptically to restore the cultures to their original volumes before they were subcultured. The cultures in the tubes showed no evidence of growth in subcultures made after a period of 186 to 224 days. The flasks, after aging 227 days, were tested for viable organisms on January 11, 1934. Three of the flasks showed no growth in the subcultures. The fourth flask yielded about 500 colonies on the infusion agar plate. Some of the colonies were as large as 3 mm. in diameter, while about 25 per cent of the colonies varied from 0.13 to 0.2 mm. in diameter. The large and small colonies, when subcultured to blood infusion agar, maintained their relative sizes and both were smooth in appearance. The strains have been maintained on blood infusion agar slants and designated as O3-64 large and O3-64 small, respectively. The O3-64 large colony strain frequently has shown a mixture of large and small colony types upon plates which were made from old cultures. However, the organisms in the large colony type grow much more rapidly than do the organisms in the small colony type, so, if an old culture of the large colony type strain which might be showing a mixture of the two colony types is subcultured a few times at fairly short intervals, the resulting culture shows only the large colony type. The O3-64 small colony type strain has been found to be very stable. It has been maintained on blood infusion agar for over 6 years and the colonies are no larger than when first isolated in January, 1934. It is this dwarf colony type O3-64, derived from Park 8 strain which has been used in the experiments which follow for comparison with organisms from other colony types.

Both the O3-64 large and the O3-64 small colony strains fermented glucose and dextrin but not sucrose. The small colony strain, however, required a longer time to bring about the reaction. Both colony types were pathogenic for guinea pigs. The large colony type killed each of two guinea pigs in less than 24 hours. The small colony type, when injected into two guinea pigs produced death in slightly less than 70 hours and 10 days, respectively. Both strains were agglutinated to the same titer by immune rabbit serum which had been prepared against the parent Park 8 strain.

When it was observed that this 225-day-old broth culture yielded both large and small colony types upon plating, the contents of the flask were filtered through a sterile Berkefeld N filter employing a negative pressure of 9 cm. of mercury. There was no evidence of bacterial growth ever taking place in the filtrate, as the filtrate remained perfectly clear for a period of about 5 months at room temperature. Various portions of the filtrate were transferred to fresh infusion broth and incubated at 37°C. but these failed to show any evidence of bacterial growth during the 5 months' period. At various times several drops of the clear filtrate were transferred to infusion agar plates and incubated at 37°C. but these also failed to show evidence of bacterial growth as long as they were held under observation. On various occasions several drops of the clear filtrate were transferred to infusion agar plates and after a few days incubation at 37°C. the surface of the plates was washed off with sterile infusion broth and the washings transferred to another fresh infusion agar plate by the Hauduroy technic. These serial plate washings also failed to bring forth bacterial growth. From this experiment it was concluded that the organisms in neither the large nor the small colony forms contained in the 225-day-old culture were filterable.

Another series of broth tubes, 9 in number, were inoculated with a single cell culture derived from one of the Park 8 cultures (intermediate type) and, after incubation, the cultures were allowed to age at room temperature and in the dark. About once

a month sterile distilled water was added aseptically to the tubes to replace that lost by evaporation. From time to time, 3 drops of the aging culture were transferred to the surface of a fresh, sterile, infusion agar plate and the inoculum spread over the surface of the plate with a sterile glass spreader. Viable organisms were isolated from the cultures after they had aged 483 days but not after 607 days. Upon one occasion one of the tubes yielded SR, R and the small colony forms. A small colony was isolated and verified as a small colony variant of the diphtheria organism. The culture appeared to be identical with the O3-64 small colony variant.

Upon numerous other occasions small colonies have been encountered upon plates along with the larger size diphtheria colonies.

MACROSCOPIC APPEARANCES OF THE DWARF TYPE COLONIES

On infusion agar. The dwarf type colony is very much smaller than the S, SR or R types. The size of the individual colonies ranges from those colonies barely visible to the naked eye to colonies 0.2 mm. in diameter. They are thus about one-tenth to one-thirtieth the diameter of the S or *mitis* colonies. The size of the colonies does not increase appreciably upon aging nor by repeated cultivation on satisfactory culture media. The colonies are visible to the naked eye if the plate is examined carefully under proper conditions of light. The colonies are convex, round and the margins even. By transmitted light the colonies are opaque.

On potassium tellurite chocolate agar. The colonies have the same general appearance as on plain infusion agar. At first they are colorless or grayish, sometimes becoming small black specks as the culture ages, at other times failing to reduce the tellurite.

In infusion broth. The small colonies have been observed to grow in this medium with a faint turbidity which is slightly granular. They have never been observed to grow in the form of a pellicle, which is so characteristic of the SR type, nor as a gran-

ular sediment with clear supernatant, so characteristic of the R type. They have given uniform suspensions in physiological salt solution.

PRODUCTION AND DESCRIPTION OF THE G (GONIDIAL) COLONY TYPE

Growth was obtained from filtrates of diphtheria cultures at various times during these studies and under various circumstances. Since the bacterial growth fulfilled the criteria for filterability and the G culture phase, the colonies have been designated as G colonies.

1. *Isolation of G colonies from a highly acid broth tube.* In the experiment reported in table 1 the pH 5.6 tube showed no evidence of growth, whatsoever, during the 115-hour period of incubation. When three drops from this tube were transferred to a glycerol agar plate, pH 7.2 and incubated three days, a large number of very small colonies appeared. This was the first instance in which a "culture," which showed no visible evidence of bacterial growth, yielded numerous, very small colonies upon plating.

The pH 5.7 glycerol broth tube in the same series had shown, after 115 hours incubation, a very small speck of growth floating on the surface. When three drops from this tube were transferred to glycerol agar, pH 7.2, only three colonies of normal size appeared among many very small colonies. The contents of the tube were then filtered through a sterile Berkefeld N filter. This tube was chosen because it contained both the large and small colony forms. No bacterial growth appeared in the filtrate as long as it was kept under observation. Likewise, no growth occurred in serial broth 'cultures' made from the filtrate. After the filtrate had aged three weeks, two drops were seeded onto the surface of a glycerol agar plate. This plate showed no signs of growth after 4 days' incubation, at which time the surface was washed off with a few drops of sterile glycerol broth and the washings transferred to a second sterile glycerol agar plate, by the Hauduroy technic. This second plate, after three days' incubation, showed numerous very small colonies. These small colonies were called "G" colonies by Dr. Philip Hadley.

Portions of the filtrate were sealed off in ampoules and these failed to give any evidence of growth when similarly treated two years later.

Some of these small colonies were picked off to glycerol broth and to a glycerol agar slant. The growth in broth was carried along for 16 culture generations over a period of approximately 9 months. It always grew as a + or ++ fine granular sediment with an occasional light clouding of the broth. At this point a broth culture was sealed in an ampoule, and when opened one year and eight months later the organisms were no longer viable.

The organisms grew on the glycerol agar slants as small, raised, smooth colonies. On the 6th glycerol agar slant in the series, the largest colonies measured $324\ \mu$ in diameter. The largest colonies ever observed in subcultures of this strain were one millimeter in diameter. The organisms making up these very small colonies were gram-positive diplo-rods about one micron in length and about 0.2 micron in width. Occasionally the size of the organisms varied from cocci of about $0.5\ \mu$ to rods of the A2 type (Westbrook, 1900) which were $1.5\ \mu$ in length. When transferred to glycerol broth, the organisms grew as a somewhat viscid sediment. Upon shaking the tube, one portion of the sediment remained attached to the bottom of the tube while the remainder swirled up into the liquid in a delicate spiral. The organisms did not ferment glucose, maltose, sucrose, lactose, galactose or glycerol, using brom-cresol purple as the indicator. When the cultures were tested with the quinhydrone pH indicator, the change in pH was found to be not greater than 0.25 pH, showing that the substances were not fermented. A glycerol broth culture sealed in an ampoule remained viable for 17 months but on other occasions the strain was not viable in ampoules 15 or 20 months old. The organisms were non-pathogenic for guinea pigs.

This strain, called F1, was kept under observation for 39 culture generations, covering a period of three and one-half years, without any change from the characteristics indicated above. The strain became contaminated with a mold during the summer vacation and all subcultures from previous transplants on hand

failed to grow. For this reason, observations on this strain could no longer be continued.

No such results were obtainable with the tubes of a very alkaline reaction, namely those at the opposite end of the series cited in table 1. Serial transfer of the Park 8 strain in alkaline broth, likewise, brought about no change in colony form. This is quite in contrast with the results which Hadley, Delves and Klimek (1931) found when working with the Shiga dysentery bacillus. They observed the G type of culture to be produced in many instances in alkaline infusion broth but never in acid media.

2. *Production of G colonies by serial transfer in acid broth.* Serial transfer of the Park 8 strain, Sr phase, in acid broth brought about the production of very small colonies, as mentioned previously. Plating out the growth from the 8th tube in the acid series, which contained 5 ml. of pH 6.2 broth plus two drops of sterile N/10 HCl onto a glycerol agar plate showed about 10 per cent of the colonies to be of the extremely small type. Several of these small colonies were fished by means of the low power of the microscope and sterile glass threads to tubes of pH 6.2 glycerol broth. Half of the subcultures showed visible growth; the other half did not. One of these subcultures which showed no visible growth in 5 days was plated out and, surprisingly, showed numerous small colonies. These small colonies, when subcultured to broth, imparted a ++ clouding of very fine particles in 48 hours. The broth culture was then filtered through a Berkefeld N filter, employing about 10 cm. negative pressure. The same day the filtration was made, December 5, 1930, two drops of the filtrate were seeded onto an agar plate. No growth appeared on this series of plates carried on by the Hauduroy technic. When the filtrate was replated on February 16, 1931, numerous G colonies appeared on the plate.

One of the above subculture tubes which had showed visible growth was plated and the plate showed both the large and small colony types. One of the very small colonies was transferred to broth, incubated and replated. This plate also showed large and small colonies. One of the small colonies was trans-

ferred to pH 6.2 broth and after 48 hours incubation showed a pellicle, clouding and sediment. This culture was then filtered through a Berkefeld N filter with a negative pressure of less than 10 cm. mercury. The culture before filtration was obviously a mixture of the large and small colony types. The filtrate, itself, did not show any visible signs of bacterial growth but on the second serial Hauduroy plate from the filtrate, G colonies were present. This strain, F3, was carried through 60 culture generations over a period of over 8 years without its reversion to the original large colony type. When the strain was grown for 96 hours (+ + clouding) in pH 7.3 broth to which had been added one per cent of glucose, maltose and sucrose, respectively, there was an increased acidity of 0.65 pH. In the case of lactose, galactose and glycerol, the change in pH was only 0.2 (determined by the quinhydrone pH indicator). The organisms were not pathogenic for guinea pigs.

3. *Isolation of G colonies from diphtheria toxin.* In two instances in which diphtheria toxin had been prepared by filtering 8-day-old cultures of the Park 8 strain through sterile Chamberland L3 filters, under less than 10 cm. negative pressure, G colonies were obtained by serially culturing the filtrates on glycerol agar plates according to the Hauduroy technic. In one instance the G colonies appeared on the first plate, and in the other case the G colonies appeared on the 8th plate.

4. *Production of G colonies by growth of the Park 8 strain in the presence of LiCl.* The Park 8 strain was propagated in serial passage for 15 culture generations in glycerol broth containing 0.25 per cent LiCl. The concentration of LiCl was then increased to 0.5 per cent. On the plate inoculated from the second 0.5 per cent LiCl tube there were numerous large and small colonies. Similar small colonies had appeared on earlier plates but they had been ignored since a search was being made particularly for rough colonies. Some of these small colonies were transferred to tubes of plain infusion broth in which some of the colonies produced visible growth and some did not. One of the tubes showing visible growth was seeded into a large tube of broth, incubated overnight and then filtered through a Berkefeld

N filter employing a negative pressure of 10 cm. of mercury. This filtrate, F4, was allowed to age 5 weeks and then three drops were seeded onto a glycerol agar plate. Upon incubating, this plate developed numerous very small colonies composed of small bi-polar staining rods about one micron in length. Some of these small colonies, GF4 strain, were suspended in broth and then transferred to various media, as follows:

A tube of glycerol broth, after inoculation and incubation, was sealed as an ampoule. Upon opening this ampoule two years and three months later, no visible organisms were evident.

A tube of infusion broth was inoculated and transfers made weekly to tubes of fresh broth for 27 cultural generations. Throughout the series the organisms grew in the broth as a slight turbidity with a ropy sediment. Occasionally, plates were inoculated throughout the series, but these showed only pin point colonies, about 0.2 to 0.3 mm. in diameter. The size of the cells still remained very small, being about 1.6 micron in length.

Three guinea pigs were next inoculated with the growth from the first and second broth tubes in the series. The ropy sediment was collected from the bottoms of the tubes by means of a sterile Pasteur pipette, centrifuged in a Hopkin's tube to determine the volume of the sediment and then resuspended in sterile saline. It was estimated that the suspension contained at least two billion organisms per ml.

The three guinea pigs were treated as indicated in table 2.

Another guinea pig was inoculated April 25, 1931, from the nineteenth cultural generation in the infusion broth series with all of the three-day growth from a plain agar slant. This animal appeared quite normal on May 4th, but developed paralysis within two months and died August 3rd.

A plain agar slant was inoculated and the strain propagated for 22 cultural generations. The organisms grew throughout the series as moist, pin-point colonies, about 0.2 mm. in diameter. Near the end of the series they assumed a creamy color. All of the growth from the twentieth agar slant in the series was inoculated into a 405-gram guinea pig on April 25. The animal appeared perfectly normal on May 4, but died on July 23. Like

the four other guinea pigs inoculated with this strain, the animal developed a paralysis of the posterior extremities prior to death.

Infusion broth containing one per cent glucose was inoculated and serial transfers made for 17 cultural generations. The organisms grew as a slight turbidity and a ropy sediment. The appearance and the amount of growth in the glucose broth series was practically the same as in the infusion broth series, so the series was discontinued. The last culture in the series was inoculated into a tube of infusion broth, incubated until there was good growth and then sealed as an ampoule. There were no viable organisms in the ampoule when opened three and a half years later.

TABLE 2

Effect produced in guinea pigs by the inoculation of the G74 strain

DATE		GUINEA PIG 4	GUINEA PIG 5	GUINEA PIG 6
2/ 9/31	Weight in grams.....	280	315	300
2/ 9/31	Intraperitoneal injection...	0.5 ml.	1.5 ml.	3.0 ml.
2/17/31	Weight in grams.....	315	355	310
2/17/31	Intraperitoneal injection...	0.5 ml.	1.5 ml.	4.0 ml.
2/24/31	All of the animals appeared normal			Paralysis noticed
3/ 3/31				
3/16/31			Paralysis noticed	

All of the animals died during the latter part of March.

Litmus lactose agar (Difco), was inoculated, since Hauduroy reported this medium very favorable for the growth of the filterable forms of *C. diphtheriae* and other bacteria. Serial transplants were made about twice each week for 16 cultural generations. At first the organisms produced acid on the medium and grew well. After the 9th transplant the growth became less abundant and very little acid was produced. All of the growth from a three-day-old slant was inoculated intraperitoneally into a 320-gram guinea pig on April 25. The animal appeared perfectly normal on May 4, but was found dead on July 13.

Loeffler's medium was inoculated, since this medium is so favorable for the growth of the diphtheria bacillus, but the serial

transfers were soon discontinued because the pin-point colonies were difficult to see on the white surface of the medium.

A blood-agar slant was inoculated and the strain propagated on this medium for 64 cultural generations over a period of three and one-half years. Growth from the 20th culture in the series was inoculated intraperitoneally into a 495-gram guinea pig on April 25. The animal appeared perfectly normal on May 1, but was very emaciated and paralyzed in the posterior extremities on July 17 and was sacrificed. No macroscopic lesions were observed at autopsy. The adrenals appeared normal. The growth from the 50th culture in the series was inoculated into a

TABLE 3
Effect of the aging of the filtrate on the appearance of small colonies

DATE OF FILTRATION, 1/27/31	FILTRATE SEEDED DIRECTLY TO:				
	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
	1/27/31	2/ 1/31	2/ 6/31*	2/11/31*	2/16/31*
Date on which surface of plates were washed and the washings transferred to fresh plates	2/ 1/31	2/ 6/31*	2/11/31*	2/16/31*	
	2/ 6/31*	2/11/31*	2/16/31*		
	2/11/31*	2/16/31*			
	2/16/31*				

* Plate showed visible growth of the G colonies.

285-gram guinea pig. The animal showed no thermal reaction and did not fail to show a steady gain in weight during a period of observation extending over three months.

One drop of a suspension of strain GF4 was inoculated into each of four large test tubes of infusion broth and incubated overnight. The cultures were then filtered through Chamberland L2, 3, 5, and 7 candles, respectively, employing negative pressure of 8 to 10 cm. of mercury. The filtrates were seeded immediately onto the surface of glycerol agar plates and every 5 days the plates were washed off with sterile broth by the Hauduroy technic and the washings transferred to the surface of fresh

glycerol agar plates. The L2 filtrate yielded small colonies on the second serial plate, 10 days after filtration. Filtrates from the L3, 5 and 7 candles yielded small colonies on the third serial plate, 15 days after filtration. Of special interest was the relationship of aging of the filtrate to the rapidity of the appearance of the small colonies on the plates. Table 3 summarizes the results, which are a confirmation of the results obtained by Miss Richardson, as reported by Hadley, Delves and Klimek (1931).

MORPHOLOGY AND TINCTORIAL PROPERTIES OF ORGANISMS IN THE G TYPE COLONY

In morphology the organisms in the G colonies were short, slender rods. Sometimes the cells were nearly spherical, at other times cocco-bacillary. The organisms were granular but swollen and "involution" forms were not observed. The organisms did show pallisade arrangements. Delicate skeins or filaments were not observed but mention has been made already of the fact that the organisms grew quite often as a ropy sediment.

The organisms were gram-positive, about that there was no doubt, but many of the organisms were easily decolorized by alcohol so that in each field one saw, in addition to the typically gram-positive organisms, gram-negative cells and gram-negative cells containing gram-positive granules. The organisms stained unevenly with Loeffler's methylene blue stain and Neisser's granule stain.

BEHAVIOR OF THE ORGANISMS OF THE SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONIES IN SOLUTIONS OF NaCl OF VARIOUS CONCENTRATIONS

Technic. The technic for the salt agglutination test was based on that used by Wilson (1930) in his study on *Salmonella aertrycke*.

In these studies the organisms were grown on plain infusion agar slants, or in Roux flasks containing infusion agar, for 24 hours, and washed off with about 5 ml. of distilled water; 0.2 ml. of the suspension was dispensed into each of nine agglutina-

tion tubes containing 0.8 ml. amounts of distilled water, 0.2, 0.5, 0.8, 1.0, 2.0, 3.0, 5.0 and 8.0 per cent NaCl, respectively. The contents of the tubes were well mixed by shaking, then incubated in the water bath at 55°C. for 24 hours with readings at the end of one hour, four hours and 24 hours. Very seldom was there any marked change in the readings made after the one-hour incubation period. The amount of agglutination in each tube was recorded as from + + + + to +.

Table 4 summarizes the results obtained in a typical salt agglutination test.

TABLE 4

Salt-stability of organisms of the various colony types of diphtheria bacilli

STRAIN	COLONY TYPE	CON-TROL WATER	CONCENTRATIONS OF SALINE ADDED, PER CENT							
			0.2	0.5	0.8	1.0	2.0	3.0	5.0	8.0
<i>Escherichia coli:</i>										
P3 (control) ..	Smooth	—	—	—	—	—	—	—	—	—
P94 (control)	Rough	—	—	++	+++	++++	++++	++++	++++	++++
<i>C. diphtheriae:</i>										
O3-64	Smooth	—	—	—	—	—	—	—	—	—
O3-64	Dwarf	—	—	—	—	—	—	—	—	—
II	Inter-mediate	—	++++	++++	++++	++++	—	—	—	—
II	Rough	++++	++++	++++	++++	++++	++++	++++	++++	++++
L8	Inter-mediate	+++	+++	+++	+++	+++	+++	+++	+++	+++

+ + + + = organisms completely agglutinated, — = no agglutination of the organisms.

Summary. It can be seen from the foregoing table that the behavior of the organisms in salt solutions of various concentrations of NaCl is closely related to the manner of growth in broth. The S colony phase was not agglutinated by sodium chloride in any concentration. The rough colony phase agglutinated spontaneously in distilled water and sodium chloride solutions of all concentrations. The intermediate colony phase showed partial agglutination in all concentrations of NaCl or in the lesser concentrations only. The *mitis* and *gravis* colony forms were found to be similar to the S and SR culture phases, respectively.

PATHOGENICITY OF THE ORGANISMS IN THE SMOOTH, INTER-MEDIATE, ROUGH AND DWARF COLONY PHASES

Technic for pathogenicity test. Organisms to be tested for pathogenicity were inoculated over the entire surface of suitable solid media, usually blood agar, incubated 24 to 48 hours and then washed off the media with small amounts of sterile infusion broth. The growth from one blood-agar slant was injected subcutaneously or intraperitoneally into each guinea pig of approximately 250 grams in weight. Usually a control guinea pig which had received at least 1000 units of diphtheria antitoxin was injected at the same time.

Results. Intraperitoneal injections of the S, SR, R and D forms proved fatal. Injections of the G forms brought about paralysis and death only after a period of several weeks, as previously mentioned. Control animals with antitoxin were not inoculated with the G cultures.

Upon two occasions avirulent variants were isolated from cultures of a Park 8 strain. About 25 broth cultures in test tubes of a Park 8 strain producing SR colonies were sealed off as ampoules after the cultures had shown good growth, usually after 48 hours incubation. These ampoules were stored at room temperature and in the dark. From time to time an ampoule was opened and a few drops of the contents transferred to the surface of a fresh agar plate. In the case of ampoule 096, which had aged 140 days, only one colony appeared on the surface of a glycerol agar plate. This colony was white, moist, round, convex and shiny, decidedly smoother than the colony type of the strain at the time it was sealed in the ampoule. The organisms were agglutinated by anti-Park 8 serum, produced the "*mitis*" type of colony on potassium tellurite chocolate agar but failed to kill guinea pigs weighing 285 and 369 grams, respectively.

Of greater interest is another variant of the Park 8 strain which was produced under similar conditions. Ampoule 097 was opened after 132 days and a few drops spread over the surface of a glycerol agar plate. Four colonies appeared on this plate. The colonies were distinctly smoother than the colonies produced

at the time the Park 8 culture was sealed in the ampoule. One of the colonies was transferred to a glycerol agar slant and maintained on either glycerol agar or blood agar slants as variant strain 097. The organisms gave the typical fermentations of the parent Park 8 strain, *i.e.*, acid from glucose and dextrin and no action on sucrose, and were agglutinated by anti-Park 8 serum. On December 6, 1933, the entire 48-hour growth from the surface of a blood agar slant was inoculated subcutaneously into a 574-gram guinea pig. The animal remained well. The test was repeated on January 13, 1934, using a guinea pig weighing 240 grams and this animal remained normal. It was concluded that these two variants of the Park 8 strain, 096 and 097, respectively, represented avirulent forms.

On November 20, 1934, a 326-gram guinea pig was inoculated with all the 24-hour growth from a blood agar slant of 097 and remained normal. On November 27, 1934, when the animal received a second and similar dose, the animal weighed 402 grams. The animal remained normal for ten days but was found dead on December 18 (20 days). On December 8, 1934, the 12th generation of the strain was inoculated intraperitoneally into two guinea pigs weighing 278 and 282 grams respectively, and both animals were dead within 48 hours.

This experience suggests that the Park 8 strain of the diphtheria bacillus became avirulent upon aging in ampoules but regained its virulence, as judged by guinea pig inoculations, after propagation on ordinary blood agar slants.

TOXIGENICITY OF THE ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH AND DWARF COLONY PHASES

Technic for toxigenicity test. For testing the toxigenicity of a culture, the organisms were grown for 7 to 10 days in a Roux flask containing about 100 ml. of infusion broth, pH 7.8, containing Bacto-proteose peptone. The flask was shaken to break up the pellicle, allowed to stand for a short time to allow the large clumps to settle to the bottom and the supernatant liquid decanted to a sterile filter assembly. Filtration was made through either Berkefeld or Chamberland filters by a technic which has

been described in detail elsewhere (Morton, 1938). The filtrate was placed in suitable sterile containers, a preservative, 0.3 per cent tricresol, added, and then placed in the cold room or refrigerator for a week or 10 days. After aging, the filtrate was injected in various amounts into normal 250-gram guinea pigs. The smallest amount of sterile filtrate killing a 250-gram guinea pig in 96 hours was taken as the minimum lethal dose (M.L.D.) of the filtrate.

Results. The S, SR, R and D forms elaborated a soluble toxin, as evidenced by the fact that a sterile cell-free filtrate of the cultures produced death in guinea pigs. There was, however, a quantitative difference, the D forms being much less toxigenic. Toxins from the S, SR and R forms had an M.L.D. of about 0.02 ml., whereas 1 ml. of a similar filtrate from the D form killed a 240-gram guinea pig on the eighth day. Filtrates of the G forms were not tested for toxicity since it was not possible to prepare toxin under the usual standard conditions with this culture type.

The toxin produced by two serological types was neutralized by commercial antitoxin.

AGGLUTINATION, OR AGGLUTININ ABSORPTION, OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

Technic for agglutination test. Whenever comparative tests were made the original dilution of serum in saline was prepared in sufficient amount to serve in all of the tests. The concentration of the NaCl solution was varied so as not to cause spontaneous clumping of the strains. Usually 1 per cent NaCl was employed. Saline and normal serum controls were included. The rack of tubes was placed in a water bath at 55°C. for 4 hours and then kept at room temperature overnight. Readings were made as soon as the tests were set up, after one and four hours and also after the tubes had stood overnight.

Technic for agglutinin absorption. A measured amount of serum was absorbed with generous quantities of packed, living organisms in a graduated centrifuge tube at 37°C. on three oc-

casions, which was found to be sufficient when working with sera of a titer not over 1:5120. It is not as easy as with many other organisms to produce agglutinating sera in rabbits.

Results. This part of the work has thus far only been carried far enough to establish that there are serological relationships between the several variants. The question of whether or not serological differences exist is reserved until more comprehensive experiments have been made.

The S, SR and D variants of the Park 8 strain have all been agglutinated in Park 8 antiserum to similar titer.

The two G (gonidial) strains which were tested after they had become large enough to be grown in sufficient amounts for antigens, were not found to be agglutinated by serum prepared against the parent culture.

It was impossible to employ the R variants for agglutination because of the tendency to spontaneous agglutination. However, the rough variant of the no. II strain absorbed all the agglutinins from the immune serum prepared by immunizing rabbits against the smooth form of no. II strain.

HEMOLYTIC ACTIVITY OF ORGANISMS IN THE SMOOTH, INTER-MEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

Technic for hemolysis test. Defibrinated rabbit or human blood was mixed with 10 volumes of saline and centrifuged. The supernatant fluid was pipetted off and another 10 volumes of saline added, thoroughly mixed and the suspension recentrifuged. The supernatant fluid was pipetted off and the washed blood cells resuspended in saline to make a 5 per cent suspension. For the test, itself, 0.5 ml. of fresh 5 per cent suspension of washed red blood cells was mixed with 0.5 ml. of a 48-hour broth culture of the organisms and the mixture incubated in a water bath at 37°C. for two hours. A reading was then made, the tubes kept overnight at room temperature and the final reading made next morning.

Table 5 summarizes the results obtained by testing the various colony types against the washed red blood corpuscles of rabbit and human origin.

The results shown in table 5 indicate that the greatest hemo-

lytic power is associated with the S and Sr colony types. The extreme rough colony type is slightly hemolytic and the D colony type non-hemolytic. This might account for the variations in hemolytic power of diphtheria cultures obtained by earlier workers as very little, if any, attention was paid to the colony form of the strains when tested for hemolysis.

TABLE 5

Results of testing members of the diphtheria group and different colony types of the same strain of *C. diphtheriae* against red blood cells

STRAIN AND COLONY TYPE	RABBIT RED BLOOD CELLS	HUMAN RED BLOOD CELLS
0.85 per cent saline (control).....	—	—
<i>Streptococcus hemolyticus</i> (control).....	++++	++++
<i>Streptococcus viridans</i> (control).....	A	A
<i>Corynebacterium hofmanni</i> (control).....	—	—
<i>Corynebacterium xerosis</i> (control).....	—	—
<i>C. diphtheriae</i> , O3-64 "S".....	++++	++++-
<i>C. diphtheriae</i> , O3-64 "D".....	—	—
<i>C. diphtheriae</i> , II "Sr".....	++++	++++
<i>C. diphtheriae</i> , II "R".....	++-	±
<i>C. diphtheriae</i> , II "S" derived from II "R".....	+++	++
<i>C. diphtheriae</i> , L8 "Sr".....	+++	++
<i>C. diphtheriae</i> , L8 "D".....	—	—
4 strains <i>C. diphtheriae</i> "mitis".....	++++	++++
2 strains <i>C. diphtheriae</i> "gravis".....	++	±
2 strains <i>C. diphtheriae</i> "gravis".....	+	±
5 strains <i>C. diphtheriae</i> "G".....	—	—

— = no change in the appearances of the red cells.

± = doubtful change in the appearances of the red cells.

++++ = complete hemolysis (beta type) of the red cells.

++++- = not quite complete hemolysis but more than ++, etc.

A = Alpha type of hemolysis.

FERMENTATION REACTIONS OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, DWARF AND GONIDIAL COLONY PHASES

All the diphtheria cultures tested for fermentation reactions, and this included *gravis* and *mitis* strains as well as S, SR, R and D strains, were found to produce acid from glucose and dextrin but not from sucrose. The S, SR, R and D colony types had the same fermentation reactions. There was, however, slight variation in the rate at which the acid was produced. The

SR appeared to produce acid slightly faster than the S form, but the difference was slight. There was a noticeable difference between the rate of acid production of the R as compared with the S and the SR strains, the R being much slower. The D type produced acid very much more slowly than the S, SR and R strains. The important point is that the S, SR, R and D colony variants of a strain have the same qualitative fermentation reactions, there being only difference in the rate in which acid is produced.

The G strains which were tested for fermentation reactions either failed to produce acid or else produced only a questionable amount.

RESISTANCE TO HEAT AND COLD OF ORGANISMS IN THE SMOOTH, INTERMEDIATE, ROUGH, AND DWARF COLONY PHASES

Heat. Text-books vary considerably in their statements as to the thermal death point of *C. diphtheriae*. It is generally stated that broth cultures of the diphtheria bacillus are killed by ten minutes exposure at a temperature of 58° to 70°C. One observes numerous statements in the literature which imply that the rough stage of a culture is usually more resistant to unfavorable conditions than the smooth stage. To verify that point, determinations of the resistance to a temperature of 56°C. were made on 24-hour-old broth cultures of several strains of the diphtheria bacillus as well as on different variants of the same strain.

Technic for thermal death point determination. Sterile Pasteur pipette stock was drawn into long capillary tubes of about one millimeter inside diameter and of uniform bore and walls. After flaming the tip of the capillary and allowing it to cool, the bacterial suspension was allowed to flow up into the tube by capillary attraction to a height of one inch. Air was then drawn into the capillary tube until the 1-inch column of bacterial suspension reached the 2-inch mark, then the end of the capillary was sealed in a gas flame. The capillary tube was then broken off one inch above the bacterial suspension and that end sealed in the gas flame. The sealed capillary tube, which contained a column of bacterial suspension in the middle with a column of air at

each end, was dropped into disinfecting solution to destroy any organisms which might be on the surface. A series of tubes prepared in this manner were then placed, completely submerged, in an electrically heated water bath at 56°C. and at various intervals of time a tube was taken from the water bath, immersed for a few seconds in tap water at room temperature and then placed in a rack. After the last tube in the series had been removed from the water bath the contents of each tube were transferred to a tube of sterile broth. This was accomplished by wiping the outside of the capillary tube with alcohol, allowing the

TABLE 6

Thermal death point determinations of several strains and different colony types of C. diphtheriae

STRAIN	COLONY TYPE	CON- TROL	TIME IN MINUTES AT 56°C.					
			1	2	3	4	5	6
<i>C. diphtheriae</i> , L8.....	Sr	+	+	—	—	—	—	—
<i>C. diphtheriae</i> , L8.....	D	+	+	—	—	—	—	—
<i>C. diphtheriae</i> , O3.....	Sr	+	+	+	—	—	—	—
<i>C. diphtheriae</i> , O3.....	D	+	+	+	—	—	—	—
<i>C. diphtheriae</i> , II.....	Sr	+	+	+	+	—	—	—
<i>C. diphtheriae</i> , II.....	R	+	+	+	+	—	—	—
<i>C. diphtheriae</i> , O5.....	Sr	+	+	—	—	—	—	—
<i>C. diphtheriae</i> , IX.....	S	+	+	+	+	—	—	—
<i>C. diphtheriae</i> , XII.....	Sr	+	+	+	+	—	—	—
<i>C. diphtheriae</i> , VIII.....	S	+	+	+	—	—	—	—
<i>C. diphtheriae</i> , XI.....	Sr	+	+	+	—	—	—	—

+ = growth.

— = no growth.

alcohol to evaporate, breaking one end of the tube close to the column of bacterial suspension and forcing the suspension into a tube of sterile infusion broth by gently warming the end of the capillary tube which contained the entrapped air. The tubes inoculated with the heated suspension were incubated for 96 hours, with readings being made at the end of each 24-hour period. Usually four series of tubes were carried through the operations at one time, so that all the factors in the experiment except the culture under test were as uniform as possible.

Results. Table 6 summarizes the results obtained in the ther-

mal death point determination of various diphtheria strains and also of the various colony forms of the same strain.

It can be seen from this table that the diphtheria bacillus is not very resistant to heat, being killed by one to three minutes exposure at 56°C. The slight variations in resistance to heat appear to be associated with the individual strains rather than with the colony forms of the strains. These findings are not in agreement with those of Weiland and Leinbrock (1938).

Cold. Since it is fairly generally known that the diphtheria bacillus is less effected by cold than by heat it was decided to try the S and R variants of the same strain against low temperature for varying periods of time. A knowledge of resistance of diphtheria bacilli to cold is also essential for the preservation of the organisms, since recent trends in the preservation of bacterial cultures are to employ methods of drying cultures from the frozen state.

A freezing mixture of solid carbon dioxide (Dry-Ice) and methylcellosolve was employed. This freezing mixture gave a temperature of -60 to -75°C. One milliliter of bacterial suspension was deposited in separate sterile Pyrex glass vials. Estimation of the number of organisms in the bacterial suspensions used was made by means of agar pour plates. The Pyrex glass vials containing the suspension were immersed in the freezing mixture and after varying periods of time two vials (one containing smooth organisms and the other containing rough organisms) were removed. After the suspensions had thawed out at the room temperature, 15 ml. of agar, melted and cooled to 45°C., were added to each vial, the contents were well mixed and poured into a sterile Petri dish. It was not necessary to make serial dilutions of the suspensions after being frozen and thawed as the number of viable bacteria had been greatly reduced. The number of organisms surviving the freezing and thawing, as evidenced by the number of colonies on the agar plates, were counted and the percentage of destruction calculated (table 7).

It can be seen from the results described in this section that the rough phase of the diphtheria bacillus was no more resistant to cold under these circumstances than the smooth phase.

RESISTANCE TO AGING OF ORGANISMS IN THE SMOOTH, INTER-MEDIATE, ROUGH AND DWARF COLONY PHASES

During the course of the studies many of the cultures were inoculated into tubes of infusion broth, incubated (usually overnight) until there was good growth, and the tubes were then sealed as ampoules. It was found that the diphtheria bacillus did not live as long in the ampoules as did many other organisms. Test tubes of practically the same diameter were used and they con-

TABLE 7

Destruction of C. diphtheriae by low temperature

STRAIN	DESTRUCTION IN MINUTES AT -75°C .					
	2	5	10	20	30	60
	per cent	per cent	per cent	per cent	per cent	per cent
<i>C. diphtheriae</i> , II, S.....	76.6	77.3	79.8	83.8	74.5	77.1
<i>C. diphtheriae</i> , II, R.....	76.8	76.3	77.8	84.3	85.7	80.7

TABLE 8

Resistance of the various colony types of C. diphtheriae to aging in ampoules

STRAIN	COLONY TYPE	ALIVE*	DEAD
		days	days
<i>C. diphtheriae</i> , II.....	Very nearly S	90	107
<i>C. diphtheriae</i> , II.....	R	224	267
<i>C. diphtheriae</i> , O3.....	SR	171	231
<i>C. diphtheriae</i> , O3.....	D	467†	

* This time represents the maximum number of days in which the organisms were ever found viable.

† End of series.

tained practically the same amount of infusion broth. Usually 15 or more tubes were employed in a series. Each tube in a series was inoculated with the same size inoculum. The ampoules contained approximately 5 ml. of culture and about the same volume of air above the cultures. The ampoules were stored at room temperature and in the dark. From time to time an ampoule was opened and some of the contents was seeded to an agar plate. Whenever growth occurred it was definitely identified as *C. diphtheriae*. Table 8 summarizes the results.

It can be seen in this experiment that there was a distinct difference in the resistance to aging between the colony forms of the same strain.

REDUCTION OF NITRATES

Contrary to Bergey's Manual (1939) diphtheria cultures produced nitrites when grown in the presence of KNO_3 .

CHROMOGENESIS

Various observers have reported pigmentation in certain diphtheria cultures. It has been our experience during these studies that chromogenesis took place seldom and irregularly. Upon different occasions throughout these studies it was noticed that certain strains of diphtheria bacilli developed a pigment which was somewhat out of the ordinary. A lemon-yellow pigment was observed in several cultures of one of the Park 8 strains which was in the Sr phase. In one instance the pigment was produced in a stock culture of the Park 8 strain being carried on Loeffler's medium and in the cold room. This pigmentation appeared in a few generations after receiving the strain. In another instance the lemon-yellow color developed in a single-celled strain of the Park 8 culture kept on plain infusion agar at room temperature and in diffuse daylight. It appears that the production of pigment is not necessarily associated with a change in any other characteristic of the culture. The pigmentation seems to appear more often and to a greater extent in the S or Sr culture phase.

CELL MORPHOLOGY

More significance is attached to the morphology of diphtheria organisms than in the case of any other microorganism; yet the diphtheria organism is most pleomorphic. Since attention has been directed to the study of bacterial colonies, it has been found that certain morphological forms can be associated with the various colony types. This is also true in the case of the diphtheria species.

Organisms from the S colony are fairly long and slender, sometimes slightly bent and showing the typical V, L and pallisade

arrangements. They show the classical irregular staining and granules by methylene blue. They are gram-positive.

Organisms from the SR colony are longer than the organisms from the S colonies. They show a greater tendency toward irregular staining and irregularly swollen forms. They show the typical arrangements and are gram-positive.

Organisms from the R colony are characterized by the definite tendency for the organisms to form chains or threads. They are gram-positive and usually stain more intensely than do organisms from the other colony types. The cells are usually thicker than the organisms in the S and D colony types.

Organisms from the D colonies are very short, and somewhat thick, rods. They are gram-positive and usually stain solidly with the gram and methylene blue stains. They show the typical arrangements of cells and some tendency towards the irregularly swollen forms.

Organisms from the G colonies are short rods, sometimes being nearly spherical. They show the typical arrangement of cells, are variable towards the gram stain and stain irregularly with methylene blue.

MORPHOLOGICAL FORMS NOT ASSOCIATED WITH COLONY FORM

A. *Coccoid forms*.³ At various times during these studies the bacilli were observed to assume coccus-like appearances. It was found possible to cause this change to appear or disappear at will. The change, either from rods to coccoids or *vice versa*, was in many cases practically complete. "Coccus," "coccal," "coccoid" and "staphylococcus" forms of the diphtheria bacillus have been described in the literature. Many of the circumstances bringing about these forms have been listed elsewhere (Morton).

The coccoid forms were encountered under the following conditions: In the course of the isolation of a single cell of the Park 8 strain by the method of Kahn (1929), a very rich medium was employed, consisting of infusion broth, pH 7.6, containing 5 per cent glycerol to which was added sterile glucose solution and

³ I wish to express my appreciation to Dr. Arthur Bernstein for his assistance in the study of the coccoid forms.

sterile normal rabbit serum to the concentrations of one and 20 per cent, respectively. The single cell picked was of Wesbrook's type C. Culturally and serologically the single-cell and parent strains were identical. When the single-cell strain was grown in this rich medium, only spherical cells were present; the organisms appearing very similar to staphylococci. When these spherical cells were transferred to ordinary blood agar, typical diphtheria bacilli resulted.

The single-cell culture showing typical diphtheria bacilli was seeded into glycerol-glucose-serum broth and after 24 hours the growth showed only an occasional granular rod, a few short, plump, solid-staining rods and numerous spherical cells or slightly elongated spheres. Seeded simultaneously into glycerol-glucose-serum broth and onto a Loeffler slant the resulting cultures after 48 hours incubation showed spherical cells and typical rods, respectively. The culture on Loeffler's medium contained granular, barred and solid-staining rods, whereas on blood infusion agar and glycerol agar, the culture showed the granular type of rods almost entirely.

To ascertain, if possible, which ingredient in the glycerol-glucose-serum broth was producing these variations in morphology, the following media were seeded from a Loeffler slant culture and incubated 48 hours.

Infusion broth, pH 7.3

Infusion broth + 20 per cent serum

Infusion broth + 1 per cent glucose

Infusion broth + 1 per cent glucose + 20 per cent serum

Infusion broth + 5 per cent glycerol

Infusion broth + 5 per cent glycerol + 20 per cent serum

Infusion broth + 5 per cent glycerol + 1 per cent glucose

Infusion broth + 5 per cent glycerol + 1 per cent glucose + 20 per cent serum

The media are listed according to their ability to change the rod forms into coccoid forms, the first named medium being the least effective and the last named, most effective. Ten-per-cent normal rabbit serum could be substituted for the 20 per cent with about the same results. Stained preparations were made

after the incubation period (fig. 1), then a subculture was made from each type of medium onto a Loeffler slant. The cultures on Loeffler's slants were examined after 48 hours incubation and in all cases the morphology was found to be identical with that of the Loeffler culture of the stock strain.

To determine the effect of normal serum of different species of animals on the morphology of the diphtheria bacillus, the following media were inoculated from a blood agar slant culture and incubated 48 hours.

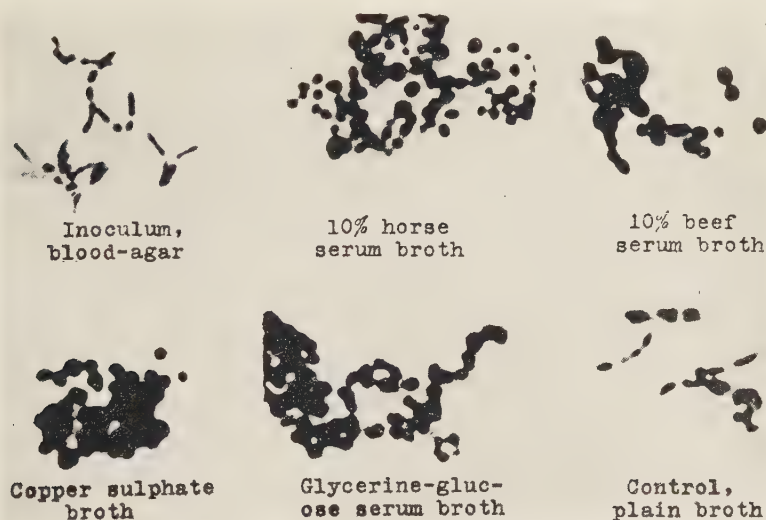


FIG. 1. *C. DIPHTHERIAE*, PARK 8, SINGLE CELLED STRAIN, GROWN 48 HOURS AT 37° C.

Methylene blue stain, $\times 2650$.

- Infusion broth + 20 per cent guinea pig serum
- Infusion broth + 20 per cent rat serum
- Infusion broth + 20 per cent human serum
- Infusion broth + 20 per cent rabbit serum
- Infusion broth + 20 per cent horse serum
- Infusion broth + 20 per cent beef serum

Practically no change in the morphology of the culture was noticed in the case of the guinea pig serum. Rat and human sera produced a slight change to the solid-staining rod and coccoid

forms, there being no difference in the effect of serum from Schick + and Schick - individuals; rabbit serum caused a distinct change to the coccoid and solid-staining forms while the horse and beef sera produced a very marked change to the coccoid forms.

No agglutinins could be demonstrated in the sera which caused the production of coccoid forms. Normal horse serum was separated into its albumin and globulin fractions by the addition of an equal volume of saturated solution of ammonium sulfate. After filtration, dialysis and sterilization by Berkefeld filtration the albumin and globulin fractions were added to the culture medium. When either fraction was added to the culture medium, coccoid forms resulted.

Best coccoid formation was obtained when the sera were added to the culture medium under aseptic conditions. If the serum and medium were autoclaved, there was very little tendency for the production of coccoid forms. Heating at 56°C. for 30 minutes had no noticeable effect on the ability of a serum to produce the coccoid forms. Normal horse serum from four different sources was tried and, while the extent of coccoid formation varied, none of the sera failed to give coccoid forms.

To test the observation of Yarisawa, that the amount of heat employed for sterilization of the Loeffler's medium had an effect on the morphology of the organisms, a batch of the medium was prepared; part of it being sterilized in the autoclave as described previously and the remainder being sterilized in the usual manner by heating in the Arnold sterilizer on three successive days. Both types of media were then inoculated from the same stock culture and incubated 48 hours. The autoclaved medium showed long, slender, pleomorphic, granular bacilli, whereas the medium which had been sterilized in the Arnold gave shorter and more solidly staining organisms.

In summary, it was concluded from these experiments that the morphology of the diphtheria bacillus may be changed to that of coccoid cells under the influence of normal sera from various species of animals. The serum from individuals of a given animal species varies somewhat in its ability to transform the

morphology to that of coccoid forms; the globulin and albumin fractions appear to work equally well in bringing about the transformation. There were no demonstrable agglutinins in sera which brought about the production of coccoid forms. The heating of a serum at 56°C. for 30 minutes did not alter its ability to transform the morphology of the diphtheria bacillus. Prolonged heating, such as autoclaving at 120°C. for 20 minutes, reduced, but did not completely destroy, the ability of serum to change the morphology of the diphtheria bacillus to that of coccoid forms.

To determine whether or not the "coccal" forms, which had been produced by Pope and Pinfield with the aid of copper sulphate, were in any way similar in appearance to the coccoid forms produced in glycerol-glucose-serum broth, the following experiment was performed.

Sterile beef infusion broth, pH 7.3, (sterilized in the usual manner) was dispensed into sterile test tubes in 5 ml. amounts. A 0.04 per cent solution of copper sulphate in distilled water was prepared and sterilized by filtering through a Berkefeld W filter using a negative pressure of 10-15 cm. of mercury. The sterile copper sulphate solution was dispensed in varying amounts into the tubes of broth to give final concentrations of 17 to 38 mgm. of copper per liter. To make sure that any change occurring in the morphology of the culture was due to the presence of the copper sulphate in the medium and not to dilution, a similar series of broth tubes was set up to which were added corresponding amounts of sterile distilled water. All the tubes were incubated 24 hours to insure sterility of the medium, then two drops of a suspension of the single-cell strain, prepared by washing off a blood-agar slant, were added to each tube. The tubes were then incubated 48 hours and stained preparations made.

In all the tubes containing the copper sulfate, the morphology of the organisms was that of coccoid and diplococcoid forms, with a few solid staining and barred forms present. The control series of tubes showed only typical, granular bacilli.

The stained preparations made of cultures from glycerol-glucose-serum broth and from copper sulphate broth appeared

practically identical. The fine granular types of growth in the two media also appeared very similar. The growth from a flask of broth containing copper in the concentration of 23 mgm. per liter was centrifuged and resuspended in 0.85 per cent solution of NaCl. The resulting suspension of coccoid forms was agglutinated by rabbit serum produced against the granular forms of Park 8 rods to the full titer of the serum. Transplants of these coccoid forms were as virulent for guinea pigs as the stock strain which was maintained on blood agar.

Pope and Pinfield attributed the "coccoid" formation as being due to the presence of the copper ion in the medium. Some observations suggested that sulphate and magnesium ions as well

TABLE 9
Effect on the morphology of C. diphtheriae of various salts in the medium

SALT	MORPHOLOGY OF CELLS WHEN 48 HOURS OLD
CuSO ₄	Coccoids
MgSO ₄	Coccoids
Na ₂ SO ₄	Coccoids and a few very short rods
(NH ₄) ₂ SO ₄	Short, solid rods in pairs and some coccoids
Cu(NO ₃) ₂	Coccoids and some very short rods
NaNO ₃	Typical rods, very granular
MgCl ₂	Coccoids and some very short rods
NaCl	Typical rods

Control: 5 ml. of broth and 2 ml. of distilled water. Typical rods.

as copper ions might produce the change in morphology. The final concentration of copper sulphate in the broth which gave good coccoid formation was N/1000. Various other salts were made up in aqueous solution, sterilized by Berkefeld filtration and added in 2 ml. amounts to tubes containing 5 ml. of broth to give a final concentration of N/1000. The plan of the experiment was essentially the same as that described for the previous experiment. Table 9 summarizes the experiment.

Cultures consisting of the coccoid forms were inoculated into plain Hiss serum water + brom-cresol purple and also into tubes of the same medium containing, respectively, glucose, sucrose, and dextrin in the amounts of one per cent. The fermentation

reactions were typical for *C. diphtheriae*. As reported by Parish, the coccoid forms persisted in the Hiss serum water. When the stock culture of *C. diphtheriae* was inoculated into a similar set of tubes of Hiss serum water + brom-cresol purple containing the three carbohydrates and a control tube of plain Hiss serum water and indicator, the growth in the control tube and in the tube containing sucrose (which was not fermented) was made up of a large number of coccoid forms and short solid-staining rods. The coccoid forms were even more numerous in the glucose and dextrin tubes in which the carbohydrates had been fermented. A tube of infusion broth pH 6.2 gave small forms, but not coccoids.

In addition to the influence of the normal sera of various species of animals upon the morphology of the diphtheria bacillus, it was concluded from these experiments that the presence of very small amounts of various salts to the culture medium also had a marked effect. In extension of the views of Pope and Pinfield, who believed that it was the copper ion in copper sulphate which caused the transformation to the coccoid forms, the sulphate ion, as well, appears to be able to bring about the transformation. It appears, then, that either the anions or the cations may influence morphology. The coccoid cells appear very much alike, whether produced by the addition of various normal sera or salts to the culture medium.

Discussion. The purpose of this section of the work has been to investigate these recorded instances in which the diphtheria bacillus assumed the coccoid form so that these transitory forms will not be confused with coccus-like variants, which appear to be entirely different in nature. The term "coccoid" is given these forms because they are very transitory, and do not take on any properties that are strikingly different from the rod forms from which they are derived and to which they readily revert. Although they may appear to be perfect spheres, it is also possible to see, mixed with them, cells which are slightly elongated and which are almost impossible to distinguish as either short, swollen rodlets or slightly elongated spherical cells.

Yarisawa's (1926) conclusion that the occurrence of coccoid

forms of *C. diphtheriae* was a transitory phenomenon in accordance with conditions, variety and surroundings of the medium apparently holds true and is borne out by the experiments in this section and literature cited previously. The change from rods to coccoids, or *vice versa*, takes place in one generation and, in many cases, is practically complete. This does not parallel the production of such dissociative variants as the rough and smooth colony types, for which a number of generations are usually required and, in which the transformation is often more gradual. Another feature in which the coccoid forms differ from actual dissociants, is that the former are not stable. Toxicity tests could not be performed because as soon as the culture was placed in a plain bouillon, suitable for toxin production, the cells assumed the rod form. Even when kept in contact with the agent which brought about their formation, the coccoids gradually reverted to the rod form. There was thus no way of maintaining a pure culture of coccoid forms suitable for prolonged study.

The fact that certain animal products, when brought into contact with the culture, exert a marked change in the morphology of the culture might explain the staphylococcus forms which Killian was able to re-isolate from guinea pigs, the change in morphology which Gins and Jermoljewa and Malcherek noticed when a sterile emulsion of guinea pigs' liver or kidney was added to the medium, and the coccoid forms which Crowell observed with the peritoneal fluid of normal guinea pigs, *in vivo*. That insufficient heating does not destroy the power of animal products to alter the morphology is in agreement with the observation that Loeffler's medium gave a variety of morphological forms (Yarisawa), depending on the amount of heat to which it had been subjected during the sterilization process; that Grubb and Koser obtained coccoid forms on liver infusion agar; that Hadley obtained coccoids by means of ascitic fluid, and that occasionally the coccoid forms appear in ordinary media (Heinemann, Parish, Parker). Pope and Pinfield's observation that the presence of a certain ion may exert a profound influence on the morphology of *C. diphtheriae* was verified. The copper ion,

however, is not the only one which can bring about this change to coccoid forms. It appears that magnesium and sulphate ions may produce this change as well. This perhaps also accounts for Maver's observations of coccoids in synthetic media.

The colony type of a culture of coccoid forms remains the same as when the culture is composed of rods. The tendency of the cells of *C. diphtheriae* to adhere to one another after fission is very striking with the coccoid forms, giving diplo and tetrad forms. Maver (1931) shows some characteristic tetrad forms from digested Loeffler's serum, which are identical to the tetrad forms observed in media containing raw serum. Plain agar plates streaked from cultures showing these tetrad forms show only colonies of typical diphtheria bacilli.

With methylene blue the coccoid cells may stain solidly or, occasionally, show one metachromatic granule within the cell. By Beck's stain the majority of organisms take the counter stain while a very few of them stain solidly or partially with gentian violet.

Grubb and Koser observed rod forms to "contract" into coccoid cells on liver infusion agar. All of these observations indicate that these coccoid forms merely represent another morphological type of the diphtheria bacillus. These coccoid forms do not assume characteristics which would place the culture, necessarily, in a colony phase different from that of the rods from which they were derived.

There are, however, instances in the literature in which the change to coccus-like cells suggests a phenomenon which is different from the production of coccoids as herein described, namely, the A-forms (Pettenkofer) of Kuhn (1924), the C-forms (coccus) of Kuhn and Sternberg (1931) and the G-forms of Hadley, Delves and Klimek (1931). In these cases the culture has become changed and stabilized in such a manner as to indicate a definite culture phase.

The C-forms of Kuhn and Sternberg and the G-forms of Hadley, Delves and Klimek are now thought to be one and the same culture phase and to represent the filterable form of the organism (Hadley, 1933). The A-forms (Pettenkofer) of Kuhn have not been

explained on the basis of colony phases. In one instance when a strain of the diphtheria bacillus was being propagated in infusion broth plus as much LiCl as the organisms would tolerate and still produce visible growth, morphological forms were encountered which were suggestive of the Pettenkofer forms. These forms were not investigated further and reverted to typical rod forms during their propagation on plain agar slants.

Kuschnarjew (1930) and more recently Stone and Hobby (1934) described "coccus" forms of the diphtheria bacillus which may be different from the coccoids just described. Cultures of these "coccus" forms in broth showed turbidity, but no pellicle. The growth near the surface was ropy. Colonies on solid media were mucoid in consistency. Stone and Hobby found their "coccoid" forms to be more susceptible to bacteriophage than the rod forms. It might be that these workers were dealing with a stage of the diphtheria culture quite different from the rod forms.

B. Changes in the morphology depending upon the youth and age of a culture. A few of the early workers were of the opinion that there was a normal evolution in the morphological forms of the diphtheria bacillus. In this connection the works of Deny (1903), Clark and Ruehl (1919), Powell (1923) and Henrici (1928) may be mentioned. All of these workers found that there was a definite series of changes in diphtheria cultures when planted on fresh media and observed over a fairly long period of time. To lend further support to the findings of the workers mentioned above, the observations which were made on the Park 8 strain during these studies might be briefly mentioned.

The strain was inoculated into glycerol infusion broth and then stained preparations were made hourly for the first 24 hours (fig. 2). Additional observations were made at the end of 36, 48, 72 and 96 hours as well as at the end of one, two, three, four, five and seven weeks. During the first few (two to five) hours of growth the cells were small and solid staining, usually appearing as very short rods or as spheres. After the 5th hour the cells elongated. Granules appeared at about the 13th hour. The

length of the rods remained fairly constant from about the 22nd hour to the end of the first week; thereafter they elongated somewhat for a period of a few weeks, but at the end of the 7th week they had shortened to coccoid forms. Club forms were first observed at 24 hours. Barred forms were first noted at the end



FIG. 2. A SERIES OF MORPHOLOGICAL FORMS OBSERVED TO TAKE PLACE AS A CULTURE OF THE PARK 8 STRAIN OF *C. DIPHTHERIAE* AGED AND AS THE AGED CULTURE RETURNED TO NORMAL GROWTH VIGOR

of the second week. About the end of the first week two general types of rods were present. One type was long and slender and stained a very faint blue with Loeffler's methylene blue, whereas the other general type of rod was short and thick and more solid staining. The granules also assumed a different appearance

when stained by methylene blue. Until the first week the granules had stained an intense blue, afterwards they were somewhat smaller and stained a bright red. These observations were made when an actively growing culture was transplanted to fresh glycerol broth and are in agreement with the observations of the workers mentioned above.

No record could be found of observations upon the series of events when an old, dormant culture was transplanted to fresh media. For these observations a broth culture of the Park 8 strain, which had aged in an ampoule for 144 days was used. Upon opening the ampoule, a Loeffler's methylene blue stain revealed that about one-half of the organisms were moderately long, slender, typical diphtheria rods of a uniform light-blue tint. The remaining rods were granular. Some of the rods possessed small granules of a deep blue color while a few of the rods contained granules of a reddish color. Sometimes these red granules appeared well separated from any bacterial cell and at other times some of the granules appeared to have a small amount of bluish debris adhering to them. The isolated granules varied in size from the limit of visibility to 0.5 of a micron in diameter. Occasionally the red granules gave a very clear-cut picture of a small diplococcus, suggesting that they might be undergoing division.

Three drops of this 144-day-old culture were spread over the surface of a glycerol agar plate and the plate incubated. Nothing appeared on the plate during the first 48 hours. At the end of the 5th day, about 300 well separated colonies were present on the plate. These colonies varied in size from 0.2 to 0.5 mm. in diameter. The colonies were convex, opaque, of a creamy color and the margins entire. As far as colony appearances were concerned, these colonies were no different from any other colonies of the Park 8 strain, being typically Sr. All of the various-sized colonies appeared to be composed of the same type of organisms.

The cells from several of the typical diphtheria colonies were stained by methylene blue. The organisms were variable in size and shape. There were solid-staining blue spheres and some

elongated spheres. Some of the elongated spheres had a light-staining band in the center so that they appeared like a diplococcus. Some of these blue cocci contained red granules within them, in other cases the small red granule protruded from the surface. In addition to these various spherically shaped cells there were a few short solid-staining rods and sometimes these short rods stained very lightly and contained a reddish granule at each end of the rod. These organisms did not at all resemble diphtheria organisms. One of the colonies showing these peculiarly shaped organisms was transferred to a glycerol agar slant. There was good growth after 48 hours and the organisms in a

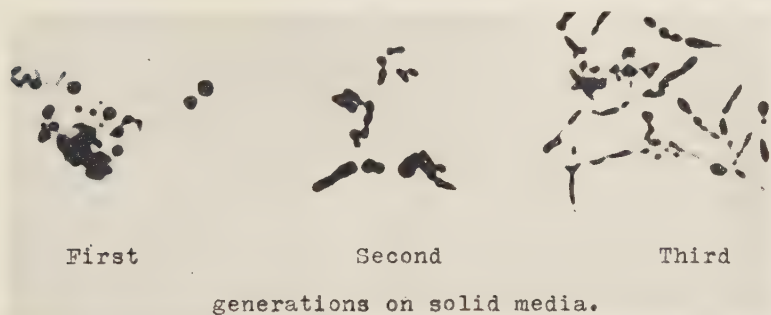


FIG. 3. *C. DIPHThERIAE*, PARK 8 STRAIN

Aged 144 days in an ampoule at room temperature then transferred to glycerol infusion agar. Methylene blue stain. $\times 2650$.

methylene-blue stained preparation still showed a very few of the spherically shaped cells, but for the most part the cells were of the rod form. There were long thick rods, much larger than ordinary diphtheria bacilli, which contained both bars and granules and short rods which were thicker than the usual diphtheria rod. The width of these short rods was about the same as the diameter of the cocci in the previous culture so that they appeared as if they were elongations of the spheres. Third and fourth generations of this strain showed the organisms in practically their normal morphology, as shown in figures 2 and 3. The growth from a 24-hour-old blood agar slant culture killed a 300-gram guinea pig in less than 45 hours. The organisms were

agglutinated by an agglutinating serum which had been prepared against the parent Park 8 strain.

At the same time that the glycerol agar plate was inoculated with three drops of the aged culture from the ampoule, three drops were also inoculated into glycerol infusion broth. The culture was propagated serially in glycerol infusion broth and showed the same transition in morphology as the culture did on glycerol agar. Only one difference was noted and that was that about twice as many generations were required in broth as on agar before the organisms returned to their usual morphology.

Thus, the granular-barred form described by Schultz was encountered. Upon other occasions it was observed in stained preparations of the diphtheria organisms, but it is not as common as Westbrook's types. The fact that this form does occur warrants its inclusion in any table of morphological types of the organism. Since coccoid forms also have been observed by numerous workers, this form, too, should be included among the morphological forms.

DISCUSSION

Inasmuch as smooth and rough colony variants exist for practically all species of microorganisms which have been studied at all extensively and since the terms *smooth* and *rough* are descriptive terms for such colony variants, have been found applicable to bacterial species in general and have priority to the terms *mitis* and *gravis* in reference to the diphtherial species, it is logical to attempt to explain the *mitis* and *gravis* variants in terms of the older and more generally used terminology. From the literature reviewed and the experimental work cited it appears that the *mitis* colonies are similar to the previously described smooth colonies for the diphtherial species, and the *gravis* colonies are similar to the larger, intermediate colony form. The group of colonies not classifiable as typically *mitis* and *gravis* appear to be nearer the rough phase than the larger intermediate or SR type colony.

A special medium containing potassium tellurite is not necessary for differentiation of the colony types. Unquestionably the

special tellurite medium may be very helpful when working with clinical material where one has present a mixture of microorganisms, some reducing the metal and the colonies thus becoming blackened, and others not reducing the metal and the colonies remaining uncolored. Anderson, Happold, McLeod and Thomson stated as one of the characteristics of the *mitis* type of colonies that the organisms are partially inhibited in their growth on the special potassium tellurite chocolate agar. This inhibition of growth of the *mitis* and of the S colony types has been observed—sometimes complete inhibition even when large inocula were used—so the question arises as to how many *mitis* or S strains might be missed when employing this medium to the exclusion of all others when searching for the diphtheria organism. In the study of 57 bacteriologically proven cases of diphtheria, Cooper, Peters and Wiseman (1939) reported the isolation of 4 strains of *C. diphtheriae* on Loeffler's medium which failed to appear on the potassium tellurite agar medium, Perry and Petran (1939) also report similar experiences. Their recommendation of the use of both media for the isolation of the diphtheria organism from clinical material is logical. A medium, in order to be perfectly satisfactory for the growth of the diphtheria organism and especially for isolating the organism from clinical material, should not exert an inhibitory action on any culture phase of the organism.

From the meager descriptions of the small colony forms in the literature it appears that the organisms are much smaller than those which are usually considered normal for the diphtherial species, and in the few instances in which the cultural reactions have been investigated, these organisms appear to require a somewhat longer period of time to bring about the various reactions which are usually considered characteristic of the diphtherial species. The observations on the strains of small colony variants described in this paper substantiate these earlier observations.

In 1935 the term "D," or dwarf, was employed (Morton, 1935b) to denote small colonies of the diphtheria bacillus because the organisms in these dwarf colonies had not been shown to be filterable, had not gone through a stage in which their growth

was invisible, had not been obtained by any special bacteriological technics and did not show a great variation from the parent culture of normal, large size except for their relatively small colony size and slowness in bringing about biochemical reactions. It was considered best to discontinue the use of the term "small" as sooner or later an abbreviation would be used, and since the letter S already signified the smooth colony type. The small colonies sometimes observed in other species of microorganisms have been designated as midget, minute or dwarf colonies. The term midget and minute, while descriptive terms for the colonies, are unsatisfactory as sooner or later they will be abbreviated to the letter M which already denotes the mucoid colony form. Neither are these small colonies the C forms of Kuhn and Sternberg (1931) as the morphology of the cells does not resemble a coccus. Moreover, Hadley (1933) announced that his G colonies were similar to the C colonies of Kuhn and Sternberg. Since the size of the colonies and the physiological activity of the organisms within the small colonies are dwarfed in comparison to that which has been commonly recognized for the species, the term D or dwarf was selected. It signifies that the size and activity of the organisms in this culture phase appear small or slight in comparison to the colony sizes and activities of those organisms commonly regarded as the normal size for the diphtherial species. Also the letter D was not being employed for any other culture phase. A later search of the literature for evidence of similar small colony forms for other bacterial species revealed that Eisenberg in 1914 also employed the term dwarf to designate a small colony form of the typhoid bacillus. This ability to exist in an extremely small colony form in addition to the more common large colony forms, is not a characteristic of the diphtherial species alone but is shared by about twenty other bacterial species (Morton).

In 1931 Hadley, Delves and Klimek described at some length the filterable form or G culture phase of the Shiga dysentery bacillus and analogous forms of culture in 11 other species, namely: *Escherichia coli*, *Eberthella typhosa*, *Salmonella paratyphi* A and B, *Salmonella enteritidis*, *Salmonella cholerae-suis*, *Sal-*

monella typhimurium I, *Salmonella typhimurium* II (*Bacillus pestiscaviae*), *Corynebacterium diphtheriae* (Park 8 strain), *Lactobacillus acidophilus* and *Vibrio cholerae*. In the same year Kuhn and Sternberg (1931) described several culture phases for bacteria, some of which were similar to the culture phases previously described by other workers. Hadley (1933) pointed out this similarity, stating that the C (Kokken formen) seemed definitely related to the G forms. In addition to producing C forms for *Escherichia coli*, *Eberthella typhosa*, *Salmonella typhimurium*, and *Corynebacterium diphtheriae*, for which Hadley, Delves and Klimek had described G forms, Kuhn and Sternberg produced C forms also from *Vibrio metchnikovi*, *Spirillum volutans*, *Bacillus anthracis*, *Bacillus pseudo-anthraxis*, *Proteus vulgaris* and *Proteus* X-19, *Mycobacterium tuberculosis*, *Salmonella suipestifer*, "Schweineseuche" and the dysentery bacillus of Shiga-Kruse.

Since 1931 small colonies and G colonies have been described for many bacterial species. Many of the small colonies referred to in the literature as G colonies are so named solely because of their small size; however, smallness of size is not the only criterion for the G culture phase. The small colonies of the diphtheria bacillus herein described as G colonies are so named because they fulfill the 5 postulates of Hadley (1931) for filterability and the G culture phase. This work is a confirmation of the experiments of Hadley and Richardson on the Park 8 strain which were described in the monograph by Hadley, Delves and Klimek. Haddow (1938) described G forms for *Salmonella paratyphi* B (Tidy) and reported the appearance of similar G colonies in cultures of *Eberthella typhosus* (Cole), *Salmonella paratyphi* A (Schottmüller) and a recently isolated strain of *Escherichia coli*. The G colonies for *Salmonella paratyphi* B (Tidy) fulfill the postulates of Hadley.

GENERAL CONCLUSIONS

A study of the diphtherial organism from many angles of approach reveals that its colonies on agar are characterized by at least four distinct, stable colony types and are not necessarily "small, grayish, granular, almost transparent, lace-like, margin

irregular" as described in Bergey's Manual for Determinative Bacteriology. As in other bacterial species, these colony types have been described as smooth (S), intermediate (SR), rough (R) and dwarf (D), according to their appearance on plain infusion agar.

Organisms from these four main colony types are true diphtheria bacilli, as judged by their tinctorial, cultural, and serological reactions and by their pathogenicity for guinea pigs.

With these differences in colonial appearances on plain agar can be associated variations in the manner of growth in liquid media, in stability in saline solutions of varying concentrations of sodium chloride, differences in appearance on potassium tellurite agar and certain quantitative variations in such properties as fermentation, toxigenicity, hemolysis, resistance to aging, etc.

There is order in the variation process within the diphtherial species. It is possible to predict, with a reasonable degree of certainty, the cultural reactions and the morphology of the organisms when a strain passes from one colony form to another. There is less certainty, however, which colony form will be the first to appear when a strain is undergoing forced dissociation. In contrast to some bacterial species, the variations in the characteristics of the organisms from the various colony forms are quantitative rather than qualitative.

In addition to the four stable colony forms of the diphtheria organism mentioned above, another phase, namely the G, was encountered. This G culture phase differs from all the other colony variants in that it is filterable, usually passes through a period of invisible growth, and when G colonies are obtained, by special culturing technics, the organisms differ from the more common colony forms biochemically, serologically and in pathogenicity. The characteristics of the G colonies of the diphtheria organism in relation to the larger and more common colony forms are not at all unlike the characteristics of the G colonies in relation to the larger colony forms of other species for which the G culture phase has been produced.

Unlike most other bacterial species the morphology of the diphtherial organism is very alterable without there being a

concomitant change in colony form. Because of its practical importance in diagnosis this aspect has been investigated quite extensively. The morphology of the cells differs with the physiological youth and age of the culture, the pH and composition of the medium, the manner in which the medium was sterilized, oxygen tension, symbiotic relationship with other organisms, and in different sections of the same colony.

Many discrepancies within the species reported by earlier workers can be explained by the quite normal variations of the diphtherial organism. These variations within the species are usually associated with the S, R, and D colony variation.

I am very grateful to Dr. Philip Hadley for his guidance and assistance in the earlier dissociative studies and to Dr. Stuart Mudd for many helpful suggestions.

REFERENCES⁴

- BECKER 1927 Ueber die Wirkung des Diphtherie-Antitoxins in vitro und in vivo. *Z. Immunitäts.*, **52**, 402-20.
- Bergey's Manual of Determinative Bacteriology. A Key for the Identification of Organisms of the Class Schizomycetes. Fifth edition, 1939. The Williams & Wilkins Company, Baltimore, Md.
- DERNBY, K. G., AND DAVID, H. 1921 A study on the preparation of diphtheria toxin. *J. Path. Bact.*, **24**, 150-9.
- DUDLEY, S. F. 1934 Discussion, Society of Medical Officers of Health. *Lancet*, **1**, 299.
- DUPRAY, M. 1924 Coagulation and sterilization of Loeffler's medium in the autoclave. *J. Bact.*, **9**, 179-81.
- GOLDSWORTHY, N. E., STILL, J. L., AND DUMARESQ, J. A. 1938 Some sources of error in the interpretation of fermentation reactions, with special reference to the effects of serum enzymes. *J. Path. Bact.*, **46**, 253-60.
- HADDOW, A. 1938 Small-colony variation in *B. paratyphosus* B (Tidy) and other bacteria, with special reference to the G type of Hadley. *J. Infectious Diseases*, **63**, 129-92.
- HADLEY, P. 1933 The relation of the bacterial variants of Kuhn to the chief phases in microbial dissociation. *J. Bact.*, **25**, 572-5.
- HEWLETT, R. T., AND KNIGHT, E. 1897 On the so-called "pseudo"-diphtheria bacillus and its relation to the Klebs-Loeffler bacillus. *Trans. Brit. Inst. Preventive Med.*, ser. 1, p. 7-32.
- HINKLEMAN, A. J. 1923 Coagulation and sterilization of Loeffler's blood serum media under steam pressure. *J. Bact.*, **8**, 315-7.

⁴ References appearing in a review article submitted to Bacteriological Reviews have not been included in this bibliography.

- JUNGEBLUT, C. W. 1928 The adaptation in vitro of diphtheria bacillus to specific antitoxin. *Proc. Soc. Exptl. Biol. Med.*, **25**, 427-9.
- KAHN, M. C. 1929 A developmental cycle of the tubercle bacillus as revealed by single-cell studies. *Am. Rev. Tuberc.*, **20**, 150-200.
- KNOX, R. 1937 The effect of serum on the colonial form of *Corynebacterium diphtheriae*. *J. Path. Bact.*, **45**, 733-8.
- KUHN, PH. 1924 Weitere Einblicke in die Entwicklung der A-formen (Pettenkoferiaformen). *Zentr. Bakt., Parasitenk. I, O.*, **93**, 280*-8*.
- KUSCHNARJEW, M. A. 1930 Variabilität des Diphtheriebazillus. *Z. Immunitäts.*, **68**, 210-17.
- MORTON, H. E. 1938 Bacterial filters and filtration technics. *Am. J. Clin. Path.*, **8**, 185-205.
- MORTON, H. E. Submitted for publication in *Bacteriological Reviews*.
- MORTON, H. E., AND CZARNETZKY, E. J. 1937 The application of sintered (fritted) glass filters to bacteriological work. *J. Bact.*, **34**, 461-4.
- MORTON, H. E., AND PULASKI, E. J. 1938 The preservation of bacterial cultures. I. *J. Bact.*, **35**, 163-83.
- WEILAND, P., AND LEINBROCK, A. 1938 Resistenzunterschiede der drei Typen des Diphtheriebacillus (*gravis*, *mitis*, *intermedius*) gegenüber schädigenden Einflüssen. *Zentr. Bakt. Parasitenk, I, O.*, **141**, 109-16.
- WILSON, G. S. 1930 The relationship between morphology, colonial appearance, agglutinability and virulence to mice of certain variants of *Bacterium aertycke*. *J. Hyg.*, **30**, 40-54.
- WILSON, H., AND GOLDSWORTHY, N. E. 1939 The use of blood agar for the identification of the types of *C. diphtheriae*. *J. Path. Bact.*, **48**, 125-8.

PLATE 1

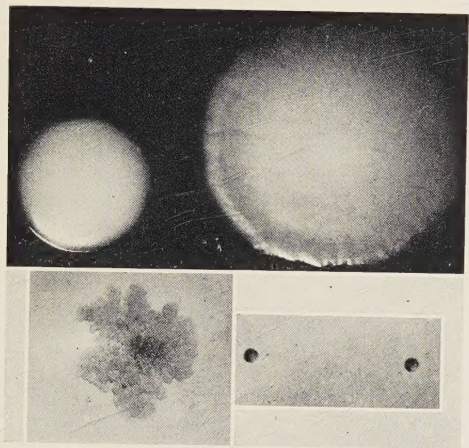
THE FOUR MAIN COLONY TYPES OF THE DIPHTHERIA BACILLUS

FIG. 1. (upper left). *S*, smooth colony, *C. diphtheriae*, Park 8 strain, grown 48 hours on infusion agar at 37°C. Reflected light. $\times 12$.

FIG. 2. (upper right). *SR*, intermediate colony, *C. diphtheriae*, Park 8 strain, grown 48 hours on infusion agar at 37°C. Reflected light. $\times 12$. These two colonies *S* and *SR*, were growing side by side on the same infusion agar plate.

FIG. 3. (lower left). *R*, rough colony, *C. diphtheriae*, II strain, grown 48 hours on infusion agar at 37°C. Transmitted light. $\times 18.8$.

FIG. 4. (lower right). *D*, dwarf colony, *C. diphtheriae*, Park 8 strain, grown 48 hours on infusion agar at 37°C. Transmitted light. $\times 18.8$.



(Harry E. Morton: Corynebacterium Diphtheriae)

